
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of:
Schulz and Romisch

Application No.: 10/566,354 Confirmation No.: 2520

Filed: August 12, 2004 Art Unit: 1656

For: PROCESS FOR PREPARING AN ALPHA-1-ANTITRYPSIN SOLUTION Examiner: Marsha M. TSAY

**THIRD-PARTY SUBMISSION IN PUBLISHED APPLICATION**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Pursuant to 37 CFR §1.99, the undersigned hereby submits copies of references with respect to U.S. Patent Application No. 10/566,354, which was published as No. 2006/194300 on August 31, 2006.

This submission is made within two months from the date of publication of the application and is accompanied by the $180.00 fee set forth in 37 C.F.R. § 1.17(p).

The seven references are identified below and a copy of each reference is attached:

1. U.S. Patent Publication No. 10/334,303, published as 2004/0124143 on July 1, 2004.

2. WO 95/35306 (PCT/US95/07616), published on Dec. 28, 1995.

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WILLIAM R. DIXON, JR.
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3. Chen, S.X et al., Purification of $\alpha_1$ Proteinase Inhibitor from Human Plasma Fraction IV-1 by Ion Exchange Chromatography, VOX Sang. 1998; 74:232-241.

4. Programme, International Meeting on Alpha-1 Antitrypsin Deficiency, (AIR 2003) World Trade Center, Barcelona Spain. June 11-13, 2003.

5. Lopez M. et al., Characterisation and Stability of a New Alpha -1 Proteinase Inhibitor Concentrate, AIR 2003, International Meeting on Alpha-1 Antitrypsin Deficiency, (AIR 2003) World Trade Center, Barcelona Spain. June 11-13, 2003.

6. Lopez, M. et al., Alpha-1 Proteinase Inhibitor; Adjustment of Individual Therapeutical Doses, AIR 2003, International Meeting on Alpha-1 Antitrypsin Deficiency, (AIR 2003) World Trade Center, Barcelona Spain. June 11-13, 2003.

7. Biescas M. et al., Viral Safety of an Alpha -1 Proteinase Inhibitor (A1PI) Therapeutic Concentrate, AIR 2003, International Meeting on Alpha-1 Antitrypsin Deficiency, (AIR 2003) World Trade Center, Barcelona Spain. June 11-13, 2003.

The cited references are believed to be relevant to the examination of the above-identified published patent application and should therefore be considered by the Examiner during prosecution.

Dated: October 30, 2006

Respectfully submitted,

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Application No. (if known): 10/566,354 PATENT

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US 20040124143A1

(19) United States

(12) Patent Application Publication (10) Pub. No.: US 2004/0124143 A1

Kee et al. (43) Pub. Date: Jul. 1, 2004

(54) METHOD FOR PURIFICATION OF ALPHA-1-ANTITRYPSIN

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(21) Appl. No.: 10/334,303

(22) Filed: Dec. 31, 2002

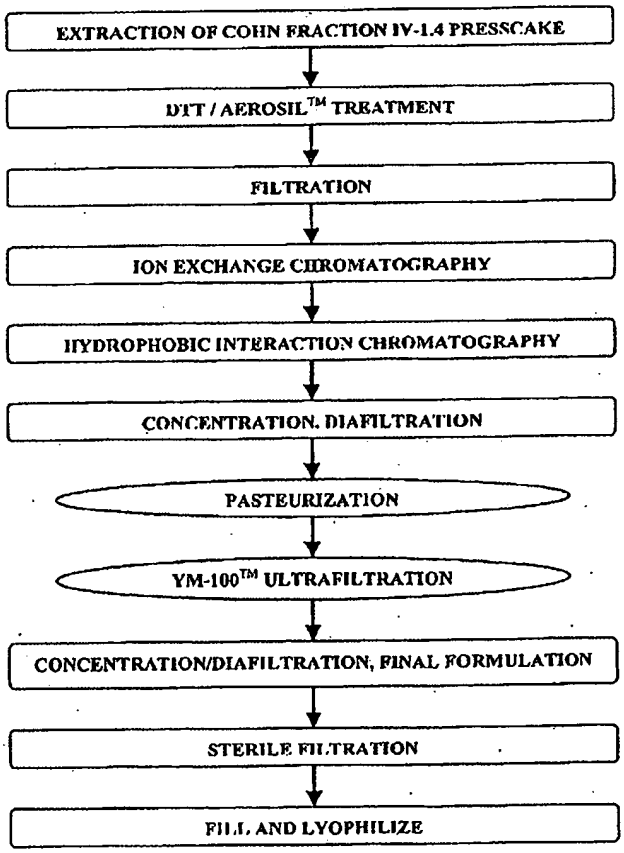
Publication Classification

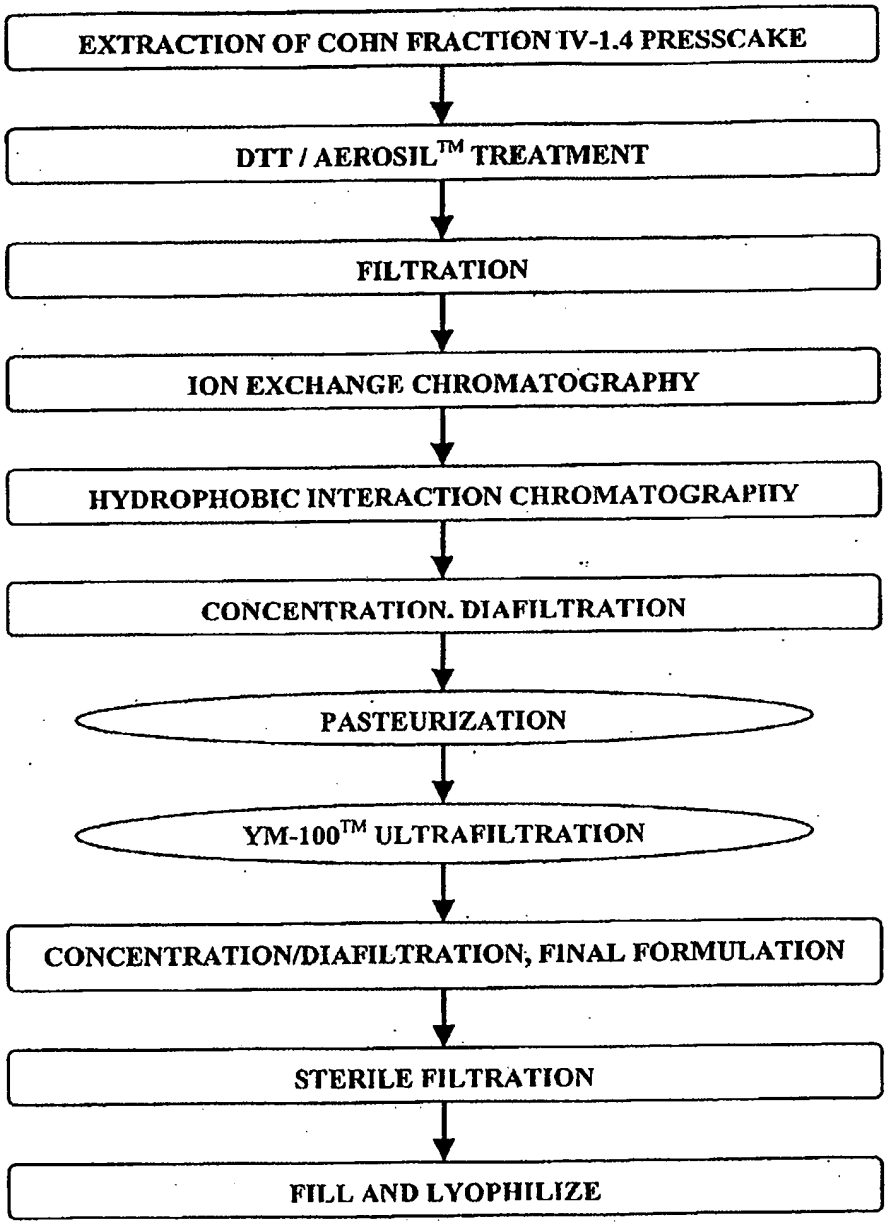
(51) Int. Cl.[7] ..... B01D 15/08

(52) U.S. Cl. ..... 210/638; 210/656; 210/757

(57) ABSTRACT

A streamlined method for purifying alpha-1-antitrypsin (AAT) from an AAT-containing protein mixture, such as a Cohn fraction IV precipitate, is provided. In the method of the invention, contaminating proteins are destabilized by cleavage of disulfide bonds with a reducing reagent, such as a dithiol, which does not affect AAT. The destabilized proteins are then preferentially adsorbed on a solid protein-adsorbing material, without the addition of a salt as a precipitant. Separation of the solid adsorbent from the solution leaves a purified AAT solution that is directly suitable for chromatographic purification, without the need for extensive desalting as in prior art processes. A process incorporating this method, which provides pharmaceutical grade AAT in high yield on a commercial scale, is also described.




EXTRACTION OF COHN FRACTION IV-1.4 PRESSCAKE
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CONCENTRATION. DIAFILTRATION
PASTEURIZATION
YM-100™ ULTRAFILTRATION
CONCENTRATION/DIAFILTRATION, FINAL FORMULATION
STERILE FILTRATION
FILL AND LYOPHILIZE

*Figure 1*

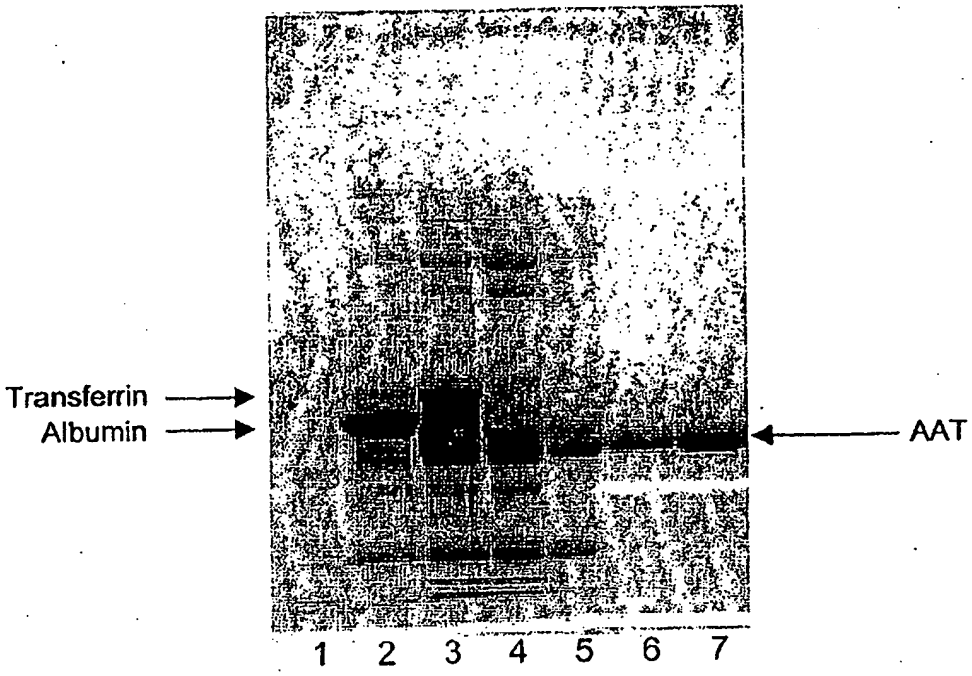


Figure 2

# METHOD FOR PURIFICATION OF ALPHA-1-ANTITRYPSIN

## FIELD OF THE INVENTION

[0001] The invention relates to protein separation and purification methods. More specifically, the invention relates to the separation of alpha-1-antitrypsin (AAT, also known as alpha-1 proteinase inhibitor, API, and $A_1$-PI) from complex protein mixtures such as blood plasma fractions, and to methods for further purification of the separated AAT so as to provide a composition suitable for pharmaceutical use.

## BACKGROUND OF THE INVENTION

[0002] Alpha-1-antitrypsin (AAT) is a glycopeptide inhibitor of proteases, and is found in human serum and other fluids. Protease inhibition by AAT is an essential component of the regulation of tissue proteolysis, and AAT deficiency is implicated in the pathology of several diseases. Individuals who inherit an alpha-1 antitrypsin deficiency, for example, have increased risk of suffering from severe early-onset emphysema, the result of unregulated destruction of lung tissue by human leukocyte elastase. The administration of exogenous human AAT has been shown to inhibit elastase and is associated with improved survival and reduction in the rate of decline of lung function in AAT-deficient patients (Crystal et al., *Am. J. Respir. Crit. Care Med.* 158:49-59 (1998); see R. Mahadeva and D. Lomas, *Thorax* 53:501-505 (1998) for a review.)

[0003] Because of its therapeutic utility, commercial AAT production has been the subject of considerable research. Much progress has been made in the production of recombinant AAT in *E. coli* (R. Bischoff et al., *Biochemistry* 30:3464-3472 (1991)), yeast (K. Kwon et al., *J. Biotechnology* 42:191-195 (1995); Bollen et al., U.S. Pat. No. 4,629,567), and plants (J. Huang et al., *Biotechnol. Prog.* 17:126-33 (2001)), and by secretion in the milk of transgenic mammals (G. Wright et al., *Biotechnology*, 9:830-834 (1991); A. L. Archibald, *Proc. Natl. Acad. Sci. USA*, 87:5178-5182 (1990)). However, isolation of AAT from human plasma is presently the most efficient practical method of obtaining AAT in quantity, and human plasma is the only FDA-approved source.

[0004] A number of processes for isolating and purifying AAT from human plasma fractions have been described, involving combinations of precipitation, adsorption, extraction, and chromatographic steps. In order to minimize the risk of pathogen transfer, pooled human plasma intended for production of human AAT for therapeutic use is screened for the hepatitis B surface antigen, and for antibodies to the human immunodeficiency virus. As an additional precaution against transmission of infectious agents, the purified product is ordinarily pasteurized by heating to 60° C. for 10 hours (Mitra et al., *Am. J. Med.* 84(sup. 6A):87-90 (1988)) and sterile filtered.

[0005] Most published processes for AAT isolation begin with one or more fractions of human plasma known as the Cohn fraction IV precipitates, e.g. Cohn fraction $IV_1$ or fraction $IV_{1-4}$, which are obtained from plasma as a paste after a series of ethanol precipitations and pH adjustments (E. J. Cohn et al., *J. Amer. Chem. Soc.*, 68:459-475 (1946)).

[0006] U.S. Pat. No. 3,301,842 describes a method for isolation of AAT from Cohn fraction $IV_1$ wherein an acridine or quinoline derivative is added to the paste in a buffer at pH 6, the precipitate is discarded, and the pH adjusted to 7.0. Additional acridine or quinoline is added, and the precipitate is collected. This precipitate is dissolved in a pH 5.0 buffer, sodium chloride is added, and the resulting precipitate discarded. The solution, containing the AAT, is further processed by methanol precipitation. Alternatively, ammonium sulfate precipitations at pH 8 and at pH 5 are conducted with plasma, with the pH 5 supernatant being further processed as above with quinoline or acridine additives.

[0007] Glaser et al., *Preparative Biochemistry*, 5:333-348 (1975), disclosed a method for isolating AAT from Cohn fraction $IV_1$ paste. The paste is stirred in a phosphate buffer at pH 8.5 in order to reactivate the AAT, which is largely deactivated by the pH of 5.2 employed in the Cohn fractionation. After dialysis and centrifugation, the supernatant is subjected to two rounds of anion exchange chromatography at pH 6.0 to 7.6 and at pH 8.6, followed by further chromatographic processing at pH 7.6 and at pH 8.0, to produce AAT in about a 30% overall yield.

[0008] M. H. Coan et al., in U.S. Pat. Nos. 4,379,087 and 4,439,358 (see also M. H. Coan et al., *Vox Sang.*, 48:333-342 (1985); M. H. Coan, *Amer. J. Med.*, 84(sup 6A):32-36 (1988); and R. H. Hein et al., *Eur. Respir. J.*, 3(sup 9):16s-20s (1990)), disclosed a procedure wherein Cohn fraction $IV_1$ paste is dissolved in a pH 6.5 to 8.5 buffer, polyethylene glycol is added, and the pH is lowered to the range of 4.6 to 5.7 to precipitate unwanted proteins. After centrifugation, AAT is isolated from the supernatant by anion exchange chromatography. Further processing provides a 45% yield of AAT with a purity of about 60%. Methods employing polyethylene glycol as a precipitant are also described in U.S. Pat. No. 4,697,003, U.S. Pat. No. 4,656,254, and Japanese patent JP 08099999, described below; and also by Hao et al., *Proc. Intl. Workshop on Technology for Protein Separation and Improvement of Blood Plasma Fractionation*, Sep. 7-9, 1977, Reston, Va.

[0009] Dubin et al., *Preparative Biochemistry*, 20:63-70 (1990), disclosed a two step chromatographic purification, in which AAT was first eluted from Blue SEPHAROSE™ and then purified by gel filtration chromatography.

[0010] Schultze and Heimburger, in U.S. Pat. No. 3,293,236, disclosed purification of AAT using cation exchange chromatography with a citrate buffer, in combination with ammonium sulfate fractionation of human plasma.

[0011] Lebing and Chen, in U.S. Pat. No. 5,610,285, disclosed a purification process which employs an initial anion exchange chromatography, followed by cation exchange chromatography at low pH and low ionic strength, to purify human AAT from plasma and plasma fractions. The cation chromatography takes advantage of the fact that active AAT does not bind to the ion exchange column under these conditions while contaminating proteins, including denatured AAT and albumin, are retained.

[0012] Jordan et al., in U.S. Pat. No. 4,749,783, described the isolation of AAT from human plasma using affinity chromatography with monoclonal antibodies. See also Podiarene et al., *Vopr. Med. Khim.* 35:96-99 (1989).

[0013] Shearer et al., in European patent application EP 0 224 811 and in the corresponding U.S. Pat. No. 4,656,254, disclosed an improved method for extracting AAT from

Cohn fraction IV paste, in which the improvement consisted of treating the paste with a larger volume of buffer, at a higher pH, than had been customary in the prior art. The combination of higher volume and higher pH increased the amount of AAT extracted from the paste. Unwanted proteins were precipitated by addition of polyethylene glycol, followed by centrifugation. An alternative procedure is disclosed, which is essentially the procedure of Coan et al., wherein after addition of polyethylene glycol, the pH is adjusted to the range of 4.6 to 5.7, and the acidified mixture held for from one to sixty minutes to further precipitate unwanted proteins. The AAT is precipitated by addition of additional polyethylene glycol, and further purified by anion exchange chromatography.

[0014] Arrighi et al., in European application EP 0717049, disclosed a process wherein fraction $IV_1$ paste is stirred in a pH 8.2 buffer at 40° C. for one hour, followed by precipitation of unwanted proteins with ammonium sulfate. The AAT is isolated from the supernatant by hydrophobic interaction chromatography at pH 7.

[0015] Kress et al., in *Preparative Biochemistry* 3:541-552 (1973), dialyzed the precipitate from an 80% ammonium sulfate treatment of human plasma, then chromatographed it on DEAE-cellulose. The product was dialyzed again and gel filtered on SPEHADEX™ G-100. AAT-containing fractions were then chromatographed on DE-52 cellulose to give AAT.

[0016] Japanese patent 59-128335 discloses the precipitation of unwanted proteins from a plasma fraction by addition of polyethylene glycol at a pH between 5 and 7, followed by anion exchange chromatography.

[0017] Bollen et al., in U.S. Pat. No. 4,629,567, disclose the isolation of AAT from a culture of yeast carrying recombinant plasmids expressing AAT. The process begins with polyethylene glycol precipitation at pH 6.5 to remove contaminating proteins, followed by anion exchange chromatography at pH 6.5 and subsequent chromatographic steps.

[0018] Dove and Mitra, in U.S. Pat. No. 4,684,723, disclose a variant of the method of Coan et al. (U.S. Pat. No. 4,379,087 and U.S. Pat. No. 4,439,358) in which AAT is purified by a process comprising the steps of (a) holding a solution containing AAT at a pH of 6.5 to 8.5 for up to 24 hours, (b) adding polyethylene glycol and an inorganic salt, so as to obtain a two-phase mixture, and (c) isolating the aqueous salt phase, which contains purified AAT.

[0019] Taniguchi et al., in PCT application WO 95/35306, disclose a similar process, involving precipitation with polyethylene glycol in the presence of zinc chloride, followed by anion-exchange chromatography and chromatography on a metal chelate resin.

[0020] Van Wietmendaele et al., in U.S. Pat. No. 4,857,317, also disclose a process for isolating AAT from the crude extract of an engineered yeast culture, which comprises addition of polyethylene glycol at pH 6.1, centrifugation to remove precipitated proteins, addition of calcium chloride, storage for 24 hours at pH 7.0, and centrifugation to further remove contaminants. AAT is then isolated from the supernatant by subsequent chromatographic steps.

[0021] Coan, in U.S. Pat. No. 4,697,003, discloses a method for isolating AAT from various Cohn plasma fractions which comprises the removal of ethanol and salts from an AAT-containing fraction, followed by anion-exchange chromatography on DEAE cellulose or a similar material under conditions such that the AAT is retained on the column while undesired proteins are eluted. Coan also describes "pasteurization" at about 60° C. or more for about 10 hours, which is stated to be sufficient to render hepatitis viruses non-infective.

[0022] Coan discloses addition of carbohydrate as a stabilization agent, either alone or with sodium citrate, in order to stabilize the AAT at the pasteurization temperature. Suitable carbohydrates are said to be mono-, di-, and trisaccharides, and sugar alcohols such as sorbitol and mannitol. AAT is prone to both polymerization and to the adoption of inactive conformations upon heating; the presence of stabilizers reduces but does not eliminate thermal inactivation (D. Lomas et al., *Eur. Resp. J.* 10:672-675 (1997)). Size-exclusion HPLC analysis has shown that 10% of monomeric AAT is polymerized or aggregated when pasteurization is carried out according to the Coan process (M. H. Coan et al., *Vox Sang.*, 48:333-342 (1985)).

[0023] T. Burnouf et al., *Vox Sang.*, 52:291-297 (1987), disclosed substantially the same procedure for isolating AAT from Kistler-Nitschmann supernatant A. DEAE chromatography of Cohn Fractions II+III and size exclusion chromatography produced an AAT which was 80-90% pure (by SDS-PAGE) with a 36-fold increase in purity. Recovery was 65-70%.

[0024] Thierry, in European patent application EP 0282363, also discloses a method of obtaining AAT from a Kistler-Nitschmann plasma fraction. Briefly, plasma is precipitated with 10% ethanol at pH 7.4, and the supernatant precipitated again with 19% ethanol at pH 5.85. The supernatant from the second precipitation is applied to a DEAE anion-exchange column, and eluted at pH 5.2 to provide AAT of about 90% purity.

[0025] Strancar et al., in PCT patent application WO 95/24428, disclose a very similar method, employing a particular class of functionalized anion-exchange media. Desalted Cohn fraction $IV_1$ is applied to the column, and contaminating proteins are eluted with low salt buffer at a pH "close to the pKa of acetic acid." (The pKa of acetic acid is 4.74.) AAT is then eluted with 50 to 300 mM NaCl at pH 7.4 to 9.2.

[0026] Japanese patent JP 08099999 discloses a method of obtaining AAT from Cohn fraction IV or $IV_1$, which involves reduction of salt concentration to below about 0.02 M, adjusting the pH to 4.5 to 5.5, and contacting the solution with a cation exchanger to adsorb contaminating proteins.

[0027] M. E. Svoboda and J. J. van Wyk, in *Meth. Enzymology*, 109:798-816 (1985), disclose acid extraction of Cohn fraction IV paste with phosphoric, formic, and acetic acids.

[0028] Glaser et al., in *Anal. Biochem.*, 124:364-371 (1982) and also in European Patent Application EP 0 067 293, disclose several variations on a method for isolating AAT from Cohn fraction $IV_1$ precipitate which comprises the steps of (a) dissolving the paste in a pH 8.5 buffer, (b) filtering, (c) adding a dithiol such as DTT, and (d) precipitation of denatured proteins with ammonium sulfate. Glaser states that the destabilized (DTT-reduced) proteins may be

precipitated by "suitable techniques such as salting, heating, change in pH, addition of solvents and the like."

[0029] Glaser et al. describe one variation in which treatment with DTT is carried out in the presence of 2.5% AEROSIL™ fumed silica, prior to precipitation with 50% saturated ammonium sulfate. Recovery of AAT was as good as it was in the absence of the silica, and the purification factor was improved by about 70%. In both references, the authors relegate the silica to a secondary role, that of an additive that improves the results of the ammonium sulfate precipitation. The effectiveness of silica alone, without ammonium sulfate precipitation, is not recognized or described. If the concentration of the protein solution appreciably exceeds about 50 mg protein/ml, AAT is reportedly lost by occlusion in the precipitate.

[0030] Ralston and Droban, in U.S. Pat. No. 6,093,804, disclose a method involving the removal of lipoproteins from an initial protein suspension via a "lipid removal agent," followed by removal of "inactive AAT" via elution from an anion-exchange medium with a citrate buffer. The lipid removal agent is stated to be MICRO CEL™ E, a synthetic hydrous calcium silicate. In the presence of a non-citrate buffer, the anion-exchange medium binds active AAT while allowing "inactive AAT" to pass through. A citrate buffer is specified for subsequent elution of the AAT from the anion exchange medium, and also for later elution from a cation-exchange medium. Ralston and Drohan do not describe the use of a disulfide-reducing agent. The process is stated to provide AAT with a product purity of >90%; and manufacturing scale yields of >70%.

[0031] W. Stephan, in *Vox Sanguinis* 20:442-457 (1971), describes the use of fumed silica to adsorb lipoproteins from human blood serum solutions. The effect of silica adsorption on the concentrations of several plasma proteins, including AAT, was evaluated, and there was no significant loss of AAT.

[0032] Mattes et al., in *Vox Sanguinis* 81:29-36 (2001), and in PCT application WO 98/56821 and published US patent application 2002/0082214, disclose a method for isolating AAT from Cohn fraction IV which involves ethanol precipitation, anion exchange chromatography, and adsorption chromatography on hydroxyapatite. The latter step is reported to remove inactive AAT, providing a product with very high specific activity.

[0033] While AAT is an effective treatment for emphysema due to alpha-1-antitrypsin deficiency, treatment is very costly (currently about $25,000 per year), due to the limited supply and a complex manufacturing process. There remains a need for more efficient and cost-effective methods for isolating human AAT from plasma and other complex protein mixtures containing AAT. In particular, ammonium sulfate precipitation followed by dialysis is a time-consuming process, that generates substantial amounts of waste water, and there is a need for scalable processes that do not require extensive dialysis while providing high yields of high activity, high purity AAT. Thermal pasteurization of AAT effectively reduces viral contamination, but it leads to the formation of inactive AAT aggregates and polymers. Thus, there is also a need for highly pure AAT with reduced viral contamination but without significant amounts of inactive AAT aggregates and polymers. The present invention addresses these needs.

## BRIEF DESCRIPTION OF THE INVENTION

[0034] The invention provides a method for purifying AAT from crude AAT-containing protein precipitates, which consists essentially of the following steps: (a) suspending the AAT-containing protein mixture in a buffer under conditions that permit the AAT to be dissolved; (b) contacting the resulting suspension with a disulfide-reducing agent to produce a reduced suspension; (c) contacting the reduced suspension with an insoluble protein-adsorbing material; and (d) removing insoluble materials from the suspension. This process provides an enriched AAT preparation, directly suitable for chromatographic processing, with reduced costs and in less time than prior art processes. Additional purification steps may be performed at the discretion of the practitioner, as described further below.

[0035] More specifically, the process comprises the steps of: (a) suspending a crude AAT-containing protein precipitate in a buffer under conditions that permit the AAT to be dissolved; (b) contacting the resulting suspension with a disulfide-reducing agent, under conditions that permit reduction of intra-protein disulfide bonds by the reducing agent, to produce a reduced suspension; (c) contacting the reduced suspension with an insoluble protein-adsorbing material, without the addition of a substantial amount of additional salts and (d) removing insoluble materials from the suspension, so as to obtain a clarified protein solution.

[0036] By "substantial amount of additional salts" is meant an amount of soluble salt or salts that will cause otherwise-soluble proteins to begin precipitating from the solution in significant amounts. Those salts ordinarily used to cause any degree of protein precipitation, in the amounts ordinarily used for such purposes, are specifically included.

[0037] The method of the invention eliminates the salting-out step which was taught by Glaser in EP 0 067 293, which in turn avoids the time and cost associated with the need to desalt the filtrate by extensive dialysis. Furthermore, the ammonium sulfate precipitation employed by Glaser limited the concentration of the protein solutions that could be processed. If the protein concentration appreciably exceeds about 50 mg/ml in Glaser's method, AAT is reportedly lost by occlusion in the Aerosil/protein precipitate. In the absence of ammonium sulfate, higher concentrations of protein should be usable without precipitation and occlusion of AAT, with associated savings in reagents and processing time, and greater throughput per batch. The process of the present invention involves two steps where protein concentration exceeds 100 mg/ml in the absence of ammonium sulfate, and no precipitation of AAT has been seen.

[0038] The combination of a disulfide-reducing agent and an insoluble protein-adsorbing material according to the invention is particularly effective at removing albumin and transferrin, which are the major protein impurities in serum-derived crude AAT preparations such as Cohn fraction IV precipitates. After removal of the protein-adsorbing material by filtration, both albumin and transferrin levels are below the detection limits of nephelometry when conducted as described herein. Further processing as described herein provides AAT with an average purity of 98% by SDS-PAGE (reduced), and high specific activity, averaging 1.06 mg functional AAT/mg. Compositions with purity greater than 99% by SDS-PAGE, and having specific activities up to 1.12 mg functional AAT/mg protein, can be obtained by the methods disclosed herein.

[0039] The crude AAT-containing protein precipitate may be derived from various sources, including but not limited to human serum, serum from a transgenic mammal that expresses human AAT, or milk from a transgenic mammal that secretes human AAT in its milk. The source is preferably serum. If the source is serum, the precipitate is preferably a Cohn fraction IV precipitate, more preferably Cohn fraction $IV_1$, and most preferably Cohn fraction $IV_{1-4}$. There are variations, known to those of skill in the art, in the method for preparing Cohn fractions, and any of them may be employed in the present invention.

[0040] The suspension buffer may be any aqueous buffer in which AAT is soluble, and is used in a volume sufficient to dissolve most or all of the AAT present in the precipitate. The preferred volume for suspension of Cohn fraction $IV_{1-4}$ is between 6 and 10 liters per kg of precipitate paste. Examples of buffers include, but are not limited to, citrate, phosphate, and Tris buffers. The preferred buffer is Tris, preferably 100 mM Tris with 20 mM NaCl. The preferred pH is between 8.80 and 8.95.

[0041] The disulfide-reducing agent may be any dithiol commonly used to reduce disulfide bonds in proteins, including but not limited to dithiothreitol (DTT), dithioerythritol (DTE), 1,2-ethanedithiol, 1,2-propanedithiol, 1,3-propanedithiol, and the like; or a phosphine such as tributylphosphine or trimethylphosphine. The disulfide-reducing agent is preferably a dithiol, and most preferably dithiothreitol.

[0042] The insoluble protein-adsorbing material may be any of various known adsorbents for hydrophobic proteins, such as fumed silica; silica hydrogels, xerogels, and aerogels; calcium, aluminum and magnesium silicates; certain clays or minerals; and mixtures thereof. Such materials are commonly used for the clarification of food oils and beverages, and are well-known to those of skill in the art. Preferably the protein-adsorbing material is a silica adsorbent, more preferably a fumed silica such as that sold under the trade name AEROSIL™.

[0043] The invention also provides a novel combination of purification and virus reduction and inactivation steps, which produces a high-safety and high-purity AAT suitable for pharmaceutical use. Specifically, while the use of dithiothreitol and fumed silica in AAT purification processes has been described previously, the combination of the two in the absence of high temperatures or a precipitating agent such as ammonium sulfate has not been described previously. Surprisingly, it has been found that the omission of a precipitating agent from a dithiothreitol-AEROSIL™ treatment step provides a highly effective purification stage. Furthermore, while the uses of dithiothreitol, AEROSIL™, anion exchange chromatography, hydrophobic interaction chromatography, pasteurization, and nanofiltration have each been previously described in the literature, these particular steps are now combined for the first time in a purification process suitable for industrial manufacture of pharmaceutical grade AAT.

[0044] The present invention provides a preparation of AAT characterized by the following properties:

[0045] (a) the alpha-1-antitrypsin contains less than 6%, preferably less than 2%, and most preferably less than 1% contaminating proteins by SDS-PAGE, and contains

[0046] (b) less than 0.1% Albumin;

[0047] (c) less than 0.8%, and preferably less than or equal to 0.2% $\alpha_1$-acid glycoprotein;

[0048] (d) less than 0.1% $\alpha_2$-macroglobulin;

[0049] (e) less than 0.1% apolipoprotein A1;

[0050] (f) less than 0.5%, and preferably less than or equal to 0.1% antithrombin III;

[0051] (g) less than 0.1% ceruloplasmin;

[0052] (h) less than 0.5%, and preferably less than 0.1% haptoglobin;

[0053] (i) less than 0.2%, and preferably less than 0.1% IgA;

[0054] (j) less than 0.1% IgG;

[0055] (k) less than 0.1%. transferrin;

[0056] (l) the specific activity of the alpha-1-antitrypsin is at least 0.99 mg functional AAT/mg, when using as an extinction coefficient $E_{1\ cm,280\ nm}^{1\%}=5.3$;

[0057] (m) less than 8%, and preferably less than 5%, of the product is of a higher molecular weight than monomeric AAT;

[0058] (n) the apparent ratio of active to antigenic AAT is greater than 1.08, preferably greater than 1.16, and most preferably greater than 1.23, when measured by endpoint nephelometry;

[0059] (o) enveloped viruses are reduced by at least 11 $\log_{10}$ units, and non-enveloped viruses by at least 6 $\log_{10}$ units, when measured in spiking studies using human and model viruses representing a wide range of physico-chemical properties; and

[0060] (p) the product is stable for at least 2 years when stored lyophilized at up to 25° C.

[0061] The apparent ratio of active to antigenic AAT in the product of the present invention is greater than unity because the purity and/or activity of the product of the present invention is greater than that of the reference standard, which is a prior art composition. Antigenic levels, as determined by endpoint nephelometry, are measured against the current protein standard (product No. OQIM15, supplied by Dade-Behring, Deerfield, Ill.), which is calibrated directly against the internationally-recognized Certified Reference Material 470 (Reference Preparation for Proteins in Human Serum; see J. T. Whicher et al., *Clin. Chem.* 40:934-938 (1994)), using reagents and AAT antibody (Dade-Behring product No. OSAz15), as supplied for the Dade-Behring Nephelometer 100.

[0062] All publications and patent applications specifically referenced herein are incorporated by reference in their entirety. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The term "AAT" refers to human AAT generally, whether heterogeneous or homogeneous, and whether isolated from human serum or from a recombinant organism. The term is intended to embrace pharmacologically effective naturally-occurring variants (see for example, Brantly et al., *Am. J. Med.* 84(sup.6A):13-31 (1988)), as well

as pharmacologically effective non-natural forms of human AAT, including but not limited to those having non-human glycosylation patterns, N-terminal methionine, or altered amino acids. Those of skill in the art will appreciate that methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, and such equivalents are anticipated to be within the scope of the invention. The preferred embodiments described below are provided by way of example only, and the scope of the invention is not limited to the particular embodiments described.

## BRIEF DESCRIPTION OF THE FIGURES

[0063] **FIG. 1** is a flow chart showing an overall AAT purification process that incorporates the present invention.

[0064] **FIG. 2** is an SDS-PAGE gel showing the proteins present in the products produced by the process of the invention at various stages. Lane 1, molecular weight markers; Lane 2, Plasma (Cryo-Poor); Lane 3, Fraction $IV_{1,4}$ Extract; Lane 4, DTT/Aerosil-Treated Extract Filtrate; Lane 5, IEC Eluate; Lane 6, HIC Effluent; Lane 7, final container.

## DETAILED DESCRIPTION OF THE INVENTION

[0065] The particular embodiment of the invention exemplified below employs a particular Cohn fraction IV paste as a starting material, but the use of similar plasma fractions is contemplated to be within the scope of the present invention. Alternative starting materials include but are not limited to other AAT-containing Cohn fractions (see U.S. Pat. No. 4,697,003), a precipitate from Kistler-Nitschmann supernatants A or A+I (P. Kistler, H. S. Nitschmann, *Vox Sang.*, 7:414-424 (1962)), and ammonium sulfate precipitates from plasma as described by Schultze et al. in U.S. Pat. No. 3,301,842. The use of protein precipitates derived from cultures of AAT-producing recombinant cells or organisms, or precipitates derived from the milk or serum of transgenic mammals, is also contemplated to be within the scope of the present invention.

[0066] There are many methods known in the art for selectively precipitating proteins from solution, such as by the addition of salts, alcohols, and polyethylene glycol, often in combination with cooling and various pH adjustments. It is anticipated that the present invention will be applicable to most AAT-containing protein precipitates containing recoverable AAT activity, regardless of how they are initially prepared. The term "crude AAT-containing protein precipitate" is used herein to refer to any AAT-containing protein precipitate prepared by one or more of these known methods, whether from serum, milk, cell culture, or other original source.

[0067] In a preferred embodiment, described below, the crude AAT-containing protein precipitate is suspended in a Tris buffer, and treated with dithiothreitol (DTT, a preferred disulfide-reducing agent) and fumed silica (a preferred protein-adsorbing material) in order to remove contaminating proteins and lipids. Where the precipitate is Cohn fraction IV, the two major protein contaminants thus removed are albumin and transferrin. DTT and other dithiols, as well as phosphines, are known in the art to reduce intrachain and inter-chain disulfide bonds. Cleavage of structurally important disulfide bonds causes partial unfolding and destabilization of those contaminating proteins that have disulfide bonds. AAT itself is not destabilized by DTT treatment because it has no intrachain disulfide bonds.

[0068] Fumed silica is known to bind preferentially to hydrophobic proteins. It is theorized that in the method of the invention, the destabilized contaminating proteins bind to a protein-adsorbing material such as fumed silica because the partial unfolding caused by disulfide bond cleavage exposes the proteins' inner core of hydrophobic residues. The scope of the invention is not limited, however, to any particular theory of operation.

[0069] In a preferred embodiment, described below, the protein-adsorbing material, together with the adsorbed contaminating proteins, lipids, and other insoluble material, is removed from the suspension by filtration so as to obtain a clarified AAT-containing protein solution. Filtration is preferably carried out with the assistance of a filtering aid such as Celite™ diatomaceous earth, and preferably the suspension is recirculated through the filter until a clarity of <10 nephelometer turbidity units (NTU)/ml is achieved. The filtrate is further processed by chromatographic techniques to afford highly pure and highly active AAT. Other methods of separation known in the art, for example centrifugation, could also be employed in place of filtration. The practitioner will select the method appropriate to the scale of operations and the nature of the protein-adsorbing material.

[0070] After removal of insoluble materials, the AAT-containing solution may be further processed by any of the methods known in the art for protein purification, particularly the methods already known to be suitable for purification of AAT. In a preferred embodiment described below, the filtrate is first subjected to ion exchange chromatography ("IEC") with salt gradient elution. The chromatography column contains an anion exchange resin which consists of a porous resin support matrix to which positively charged groups are covalently attached. These positively charged groups reversibly bind anions, including proteins with anionic groups such as AAT.

[0071] AAT, and other proteins which have a net negative charge at the pH of the eluting buffer, bind to the IEC column. Contaminating proteins having little or no negative charge pass through the anion exchange resin column without binding and exit with the column effluent. Those contaminating proteins that do bind to the column are then separated from the AAT by gradient elution. The salt concentration is gradually increased as the column is eluted in order to release sequentially the various proteins that are bound to the resin.

[0072] In a preferred embodiment, described below, the AAT-containing eluate from the IEC column is subjected to hydrophobic interaction chromatography ("HIC"). This type of chromatography employs a support matrix to which moieties are covalently attached. In an aqueous environment, these hydrophobic moieties bind reversibly to hydrophobic molecules, such as the contaminating proteins remaining in the IEC eluate. AAT is relatively non-hydrophobic, therefore the majority of the AAT flows through the column during the elution of the column with buffer, while the more hydrophobic contaminating proteins remain bound to the column. The column effluent thus contains the purified AAT. In practice, AAT has been found to have a slight affinity for certain HIC column media, and in such cases

further elution with several volumes of wash buffer may be desirable in order to recover substantially all of the AAT in the originally-applied sample.

[0073] After such additional purification steps as are required to reach the desired level of purity and activity, the AAT solution is then concentrated and sterilized. In a preferred embodiment, described below, the AAT is at a pharmaceutically acceptable level of purity and activity after the hydrophobic interaction chromatography, and no additional steps are necessary. In a preferred embodiment, described below, concentration is accomplished by ultrafiltration followed by dialysis filtration (diafiltration). In these techniques, solvent and dissolved salts and small molecules are passed through a filtering membrane, leaving behind a more concentrated protein solution. Remaining salts and small molecules in the protein solution are then exchanged with a different buffer by continuous addition of several volumes of the new buffer to the product, while maintaining a constant product volume by continuously passing solution through the same membrane.

[0074] The AAT is then provided with a pharmaceutically acceptable buffer, and lyophilized by methods known in the art, preferably by methods known to be suitable for preparing AAT therapeutic formulations.

[0075] Proteins isolated from mammalian sources may contain pathogenic viral contaminants, and it is desirable to reduce or eliminate such contamination in pharmaceutical compositions. Methods of viral reduction are known to those of skill in the relevant arts. The methods contemplated to be applicable to the present invention include, but are not limited to, pasteurization, irradiation, solvent/detergent treatment, disinfection, filtration, and treatment with supercritical fluids. Solvent/detergent treatment can be carried out, for example, by contacting a protein solution with a polyoxyethylene sorbitan ester and tributyl phosphate (see U.S. Pat. No. 4,820,805; see also WO 95/35306 for application of the method to an AAT composition.) Disinfection of a protein solution can be carried out by exposing the solution to a soluble pathogen inactivating agent, for example as disclosed in U.S. Pat. Nos. 6,106,773, 6,369,048 and 6,436,344, or by contact with an insoluble pathogen inactivating matrix, for example as disclosed in U.S. Pat. No. 6,096,216 and references therein. Filtration may be through 15-70 nm ultrafilters (e.g., VirAGard™ filters, A/G Technology Corp.; Planova™ filters, Asahi Kasei Corp.; Viresolve™ filters, Millipore Corp.; DV and Omega™ filters, Pall Corp.) Irradiation may be with ultraviolet or gamma radiation; see for example U.S. Pat. No. 6,187,572 and references therein. Inactivation of viruses by treatment with supercritical fluids is described in U.S. Pat. No. 6,465,168. Pasteurization of a protein solution may be accomplished by heating within the limits dictated by the thermal stability of the protein to be treated. In the case of AAT, pasteurization is usually accomplished by heating to about 60-70° C. In a preferred embodiment, described below, viral reduction of the AAT concentrate is carried out by pasteurization and ultrafiltration. Stabilizing additives may be added to protect the AAT from thermal degradation during the pasteurization step, as disclosed for example in U.S. Pat. No. 4,876,241. Sucrose and potassium acetate are preferably added as stabilizers, and the stabilized AAT solution is then pasteurized at about 60° C. to reduce viral contamination. The amount of sucrose is preferably at least 40%, more preferably at least 50%, and most preferably about 60% by weight. Use of less than 40% sucrose has been found to result in undesirable levels of aggregation of the AAT. The amount of potassium acetate is preferably at least 4%, more preferably at least 5%, and most preferably about 6% by weight.

[0076] After viral reduction, the AAT solution may optionally be diluted and ultrafiltered, then re-concentrated and sterilized, e.g. by filtration. The sterilized AAT-containing concentrate may then be lyophilized to form a therapeutic product. A suitable composition for preparing a lyophilized AAT powder is shown in Table 1.

TABLE 1

Composition of AAT solution for lyophilization

| Component | Function | Concentration 1.0 g/vial |
|---|---|---|
| AAT[a] | Active Ingredient | 50 mg/mL[b] |
| Sodium Phosphate[c] | Buffer, Tonicity | 20 mM |
| Sodium Chloride USP | Tonicity | 40 mM |
| Mannitol USP | Stabilizing Agent | 3% |
| Sodium Hydroxide | To adjust pH | as needed |
| Hydrochloric Acid ACS | To adjust pH | as needed |
| Water for Injection USP[d] | Diluent/Vehicle | 20 ml/vial |

[a]The final product is ≥96% AAT as determined by SDS-PAGE and ≥93% monomer by HPLC.
[b]Functional AAT activity per ml.
[c]Added as Monobasic Sodium Phosphate Monohydrate or Dibasic Sodium Phosphate.
[d]Added as Sterile Water for Injection USP.

[0077] The final formulation will depend on the viral inactivation step(s) selected and the intended mode of administration. Depending on whether the AAT is to be administered by injection, as an aerosol, or topically, the AAT may be stored as a lyophilized powder, a liquid, or a suspension. The composition shown in Table 1 is suitable for injection, and may be lyophilized and stored in glass vials for later reconstitution with sterile water. The composition of a suitable dry powder formulation for inhalation is shown in Table 2. Such a formulation is suitable for inhalation administration as described in U.S. Pat. No. 5,780,014, either with a metered dose inhaler, or with a pulmonary delivery device such as is disclosed in U.S. Pat. No. 6,138,668.

TABLE 2

Composition of AAT Formulation for Aerosol Administration

| Component | Function | Nominal Content (per unit dose) |
|---|---|---|
| AAT | Active Ingredient | 7.440 mg* |
| Sodium Citrate | Buffer | 0.059 mg |
| Citric Acid | Buffer | 0.001 mg |

*corresponds to 6 mg functional AAT, and a delivered dose of approximately 3.6 mg functional AAT.

[0078] Assays for determining the quantity and quality of AAT are known in the art and may be employed for evaluating the efficiency of the method. An example of an immunoassay involving a monoclonal antibody specific for AAT, used for measuring or detecting AAT in biological fluids, is disclosed in U.S. Pat. No. 5,114,863. An example of the use of rate nephelometry is disclosed in L. Gaidulis et al., *Clin. Chem.* 29:1838 (1983). AAT functional activity

may be assayed by measuring its elastase inhibitory capacity using a chromogenic substrate for elastase, as described in U.S. Pat. No. 4,697,003. AAT may also be assayed by measuring its trypsin inhibitory capacity in a similar manner. In a preferred embodiment, AAT is assayed by endpoint nephelometry, as described elsewhere in this specification.

[0079] The quantity of proteins may be determined by methods known in the art, for example the Bradford assay, or by absorbance at 280 nm using as an extinction coefficient $E_{1\ cm,280\ nm}^{1\%}$=5.3 (R. Pannell, D. Johnson, and J. Travis, *Biochemistry* 13:5439-5445 (1974)). SDS-PAGE with staining and densitometry may be used to assess purity of the sample and detect the presence of contaminating proteins. A reducing agent such as dithiothreitol is preferably used with SDS-PAGE to cleave any disulfide-linked polymers, thereby facilitating the comparison of total AAT to total non-AAT protein. Size-exclusion HPLC may also be used to assess purity of the sample and detect the presence of both contaminating proteins and aggregate or polymeric forms of AAT. Analysis of four lots prepared by the method of the invention showed AAT protein purity by SDS-PAGE (reduced) of at least 98%, an AAT monomer content of at least 95%, and specific activity averaging 1.06 mg functional AAT/mg protein (Table 3).

TABLE 3

| | Purity of AAT | | |
|---|---|---|---|
| Lot | % AAT Purity by SDS-PAGE (reduced) | % Monomeric AAT by HPLC | Specific Activity (mg functional AAT/mg) |
| A | 98 | 95 | 1.10 |
| B | 99 | 95 | 1.09 |
| C | 98 | 95 | 1.05 |
| D | 98 | 96 | 1.04 |

[0080] Preferred conditions for the methods of the invention are as follows:

[0081] 1. Preparation of Cohn Fraction $IV_{1-4}$.

[0082] Human plasma is cooled to −2 to 2° C. and adjusted to a pH of 6.9 to 7.5. Cold ethanol is added to a concentration of 6 to 10%, and the temperature is lowered to −4 to 0° C. The precipitate that forms ("Fraction I") is removed by centrifugation or filtration.

[0083] The filtrate or supernatant from the above procedure is adjusted to pH 6.7 to 7.1, and cold ethanol is added to a concentration of 18 to 22%. The temperature is lowered to −7 to −3° C., and the mixture is again subjected to centrifugation or filtration. The precipitate that forms ("Fraction II+III") is set aside for other purposes.

[0084] The filtrate or supernatant from the above procedure is adjusted to pH 4.9 to 5.3 and the ethanol concentration is adjusted to 16 to 20%. The temperature is adjusted to −7 to 3° C. After the suspension settles, it is adjusted to pH 5.7 to 6.1 and the ethanol concentration is adjusted to 40 to 44%. The precipitate that forms ("Fraction $IV_{1-4}$") is removed by centrifugation or filtration, and stored until needed in the form of a paste. Fraction $IC_{1-4}$ contains AAT as well as contaminating proteins and lipids.

[0085] 2. Purification with DTT and Silica.

[0086] The Fraction $IV_{1-4}$ paste is suspended in a suspension buffer (e.g., 100 mM Tris, 20 mM NaCl, pH between about 7.5 and about 9.5, preferably between about 8 and about 9) and stirred for a minimum of one hour at low temperature. The amount of buffer used ranges from 6 to 10 kg of buffer per kg of the plasma-containing fraction.

[0087] The Tris buffer suspension is then treated with dithiothreitol (DTT) and fumed silica. DTT is added to the Tris buffer suspension at a concentration in the range of about 10-50 mM. The solution is stirred for at least 30 minutes, preferably 2-4 hours, at low temperature, and preferably at a pH of about 8-9. Fumed silica is added at a concentration of approximately 100-300 g fumed silica per kg Fraction IV precipitate. The suspension is stirred for at least 30 minutes, preferably 1-4 hours, at low temperature, at a pH of about 8-9. A filter aid such as Celite™ is added at the rate of five parts filter aid one part silica, by weight, and the mixture is stirred for approximately 15 minutes. The soluble AAT product is separated from the precipitated fumed silica and contaminating proteins using a filter press, yielding the AAT final filtrate. Preferably, the suspension is recirculated through the filter press until the desired level of clarity is obtained. The AAT final filtrate is then treated further as follows.

[0088] 3. Ion Exchange Chromatography.

[0089] The AAT final filtrate is applied directly onto a chromatography column containing an anion exchange resin equilibrated with an IEC equilibration buffer. Contaminants are removed from the column by washing the column with an IEC wash buffer, and AAT is subsequently eluted using an IEC elution buffer.

[0090] 4. Hydrophobic Interaction Chromatography (HIC).

[0091] The eluate from the IEC column is prepared for HIC by adding ammonium sulfate to a final concentration of about 1 M. The solution is then filtered and applied to a hydrophobic interaction chromatography column which is equilibrated in a HIC wash buffer. Initial elution with a wash buffer provides an AAT-containing effluent, and elution with additional wash buffer removes any AAT retained on the column. The combined effluent and washes are concentrated by ultrafiltration, and diafiltered into a phosphate buffer. The final AAT concentration is preferably no greater than 7% protein.

[0092] 5. Pasteurization

[0093] The AAT concentrate is stabilized for pasteurization by the addition of sucrose and potassium acetate, and pasteurized at about 60° C. for 10-11 hours. The pasteurized solution is held at 2-8° C. pending further processing.

[0094] 6. Nanofiltration

[0095] The pasteurized AAT solution is diluted with a final formulation buffer. The diluted, pasteurized AAT solution is then filtered through two new YM-100 (Amicon) spiral-wound ultrafiltration cartridges. This nanofiltration step serves as a second primary viral reduction step. Viruses are retained by the membrane, which has a nominal 100,000 Dalton molecular weight cut-off, while AAT, which has an approximate molecular weight of 50 kD, passes through.

The AAT is collected in the permeate of the second filter and in filter post-washes. The final filtrate is collected in a bulk receiver and held at 2-8° C.

[0096] 7. Sterile filtration and lyophilization

[0097] The AAT-containing final filtrate is concentrated and diafiltered into final formulation buffer at a temperature of no more than 15° C. to form a final bulk solution. This solution is clarified and sterilized by passage through a series of sterile, bacterial-retentive filters. The sterile bulk solution is filled into sterilized glass final containers. The filled containers are freeze-dried and then sealed under vacuum.

[0098] The product is ≧96% pure AAT as determined by both SDS-PAGE and immunological assays such as ELISA or nephelometry, and is ≧93% monomer by size exclusion HPLC. The recovery based on the functionally active AAT content of the Cohn fraction IV paste is 70%.

## EXAMPLES

[0099] Fraction $IV_{1-4}$ Precipitate (667 kg) was isolated via the Cohn plasma fractionation process from 9026 liters of human plasma. The material was divided into nine batches of approximately 75 kg each. Each batch was suspended in Tris Buffer, using 6 to 10 parts buffer (w/w) relative to the presscake. The suspensions were stirred for at least 15 minutes, the temperature was adjusted to 2°-8° C., and the pH of each suspension was adjusted to 8.80-8.95 with 1 N sodium hydroxide or 1 N hydrochloric acid as necessary. The suspensions were stirred for 15 to 105 minutes (average 45 min), and monitored for protein (Bradford assay) and potency. Specific activity of each batch ranged from 0.027 to 0.045, and averaged 0.037 mg functional AAT per mg protein. Approximately 12% of the total protein was albumin, and approximately 22% was transferrin.

[0100] Dithiothreitol (DTT) was added to a final concentration of 0.01 to 0.05 M DTT (average 0.03 M) based upon the amount of Tris Buffer in each batch. After a pre-mix period of at least 15 minutes, the temperature was adjusted to 2°-8° C. and the pH re-adjusted to 8.80-8.95, and the solutions were stirred for 2 to 8 hours (average 3 hours). If necessary, the pH was again adjusted to 8.80-8.95.

[0101] Aerosil™ 380 (Degussa AG, Frankfurt-Main) was added at the rate of 13.4 to 18.6 g per liter plasma input (average 16.7 g). The suspensions were stirred for 1 to 4 hours (average 1 hour) at 2-8° C.

[0102] Celite™ 545 was added to each suspension at the rate of 5 parts Celite to 1 part Aerosil, and the suspensions were stirred at 2-8° C. Each suspension was then recirculated through a plate and frame filter press, holding 25×25 inch Cuno™ A2605-10CP filter pads (cellulose pads with inorganic filter aids; nominal cutoff 1 micron). When the turbidity was ≦10 NTU by nephelometry (minimum of 15 min.), re-circulation was discontinued and the filtrate was collected. The filter press was post-washed with TRIS extraction buffer at 2-8° C. The postwashes were combined with the initial filtrate solutions, and total protein in solution was determined by the Bradford protein assay. The filtrates were held at 2-8° C. for no longer than 19 hours. Based on AAT activity, the filtrates contained a total of 1557 g of ATT, corresponding to a 59% yield of the activity present in the original suspension of Fraction IV paste, and a purification factor of 1.5. (In view of the activity present after subsequent processing, these values appear to be low, possibly due to the presence of unidentified factors interfering with the AAT assay.) Specific activity for each of the nine batches ranged from 0.042 to 0.064, and averaged 0.056 mg functional AAT per mg protein. Albumin and transferrin were below detection limits (total protein contained less than 0.5% albumin and less than 2.5% transferrin.)

[0103] A 92-liter, 30 cm high ion exchange chromatography (IEC) column loaded with TMAE Fractogel™ (EM Industries, Hawthorne, N.Y.) was equilibrated with IEC equilibration buffer (50 mM Tris, pH 8.3-9.3, 20-25° C.). Following equilibration, conductivity of the effluent was verified to be ≦1.25 mS/cm. Each filtrate from the previous step was warmed to 20-25° C. and filtered through a Cuno Zeta Plus™ 90SP cartridge (45115-12-90SP, nominal MW cutoff of 0.1 micron) before loading onto the column with control of flow rate (≦3.0 cm/minute) and column pressure (≦20 psi). Total protein loaded onto the IEC column was limited to no more than 70% of the resin capacity. The column was then washed with five column volumes of IEC wash buffer (50 mM Tris, 25-70 mM NaCl gradient, pH 7.1-7.7) at 20-25° C., with control of flow rate (≦3.0 cm/minute) and column pressure (≦20 psi). The effluent was monitored by Bradford protein determination, assay of AAT activity, and UV absorbance at 280 nm.

[0104] AAT was eluted with approximately three column volumes of IEC elution buffer (50 mM Tris, 75-120 mM NaCl gradient, pH 7.1-7.7) at 20-25° C., with control of flow rate (≦3.0 cm/minute) and column pressure (≦20 psi). The effluent was monitored by Bradford protein determination, assay of AAT activity, and UV absorbance at 280 nm. The entire peak that eluted after application of the elution buffer was collected for further processing.

[0105] The above procedure was repeated nine times in order to process all nine batches of filtrate. Ammonium sulfate was added to the IEC eluates to a final concentration of 0.9 to 1.1 M. The resulting solutions were either used immediately, or stored at 15-25° C. for no more than seven days. Based on AAT activity, the IEC eluates contained a total of 2241 g of ATT, corresponding to an 84% yield of the activity present in the original suspension of Fraction IV paste, and a purification factor of 16.2. Specific activity for each of the nine batches ranged from 0.416 to 0.975, and averaged 0.592 mg functional AAT per mg protein.

[0106] A Cuno™ filter (Zeta Plus™ 90SP cartridge (45115-12-90SP, nominal MW cutoff of 0.1 micron) was prepared with a hot WFI flush followed by a cold WFI rinse (WFI=Water for Injection). Water was gently blown out of the filter with compressed air. Three IEC eluates, containing ammonium sulfate, were pooled and filtered through the prepared Cuno filter and subsequently combined to provide the "filtered IEC solution". The filter was post-washed with approximately 20 liters HIC wash buffer (50 mM Tris, 1 M ammonium sulfate, pH 7.1-7.7). The post-wash and the filtrate were combined and weighed. The process was repeated three times to process the nine batches of IEC eluate.

[0107] A hydrophobic interaction column (HIC) was packed with Phenyl Sepharose™ Fast Flow HS resin (Pharmacia, Piscataway, N.J.) to a volume of 49 liters (32 cm bed height), and equilibrated with HIC wash buffer (50 mM Tris,

1 M ammonium sulfate, pH 7.1-7.7). This and all column loading and subsequent elutions were carried out with control of flow rate (≦4 cm/minute), column pressure (≦20 psi), and solution temperatures (20-25° C.).

[0108] Each of the three batches of filtered IEC solution was loaded onto an HIC column. Total protein load onto the column was limited to ≦39 g protein per liter of resin. Optical density ($OD_{280}$) of the effluent was monitored, and collection was initiated when the $OD_{280}$ rose 0.04 units higher than the baseline value. The column was washed with HIC wash buffer to elute additional AAT from the column, while non-AAT contaminants remained bound to the column. Approximately ten column volumes of HIC wash buffer was applied to the column, and effluent was collected until the $A_{280}$ dropped to <0.05 units above baseline. The AAT effluent and column wash were combined and weighed. Samples were taken for Bradford protein determination, OD Protein determination, potency, and LAL (Limulus amebocyte lysate) testing. The HIC effluents were held at 15-25° C. for no more than 72 hours. Based on AAT activity, the three batches of HIC effluent contained a total of 2090 g of ATT, corresponding to a 79% yield of the activity present in the original suspension of Fraction IV paste, and a purification factor of 25.6. Specific activity for each of the three batches ranged from 0.908 to 0.986, and averaged 0.937 mg functional AAT per mg protein.

[0109] A tangential flow ultrafiltration (UF) unit containing a polyether sulfone membrane (surface area: 50 $ft^2$) with a molecular weight cut off range of 5,000-30,000 was integrity tested to ensure a bubble point of less than 1250 ml/minute. Diafiltration buffer (40 mM sodium phosphate, pH 7.2-7.6; 10 kg minimum) was recirculated through the unit for a minimum of five minutes. The recirculated buffer solution was sampled to verify proper pH (7.2-7.6) and LAL (<0.25 EU/ml). A repeat of the prewash steps was performed if pH and LAL requirements were not met. The UF unit was held for no more than 12 hours at 2-8° C. prior to HIC Effluent application.

[0110] The HIC effluent from the previous process step was mixed, and the temperature was adjusted to 15-25° C., prior to application to the ultrafiltration unit. Inlet pressure was maintained at ≦40 psi, and outlet pressure and sample weight were monitored during the concentration process. Concentration was performed until the weight of the concentrate was approximately 10 kg.

[0111] Following concentration, the HIC effluent concentrate was diafiltered, exchanging the Tris-buffered ammonium sulfate solution with a sodium phosphate buffer. Diafiltration buffer (40 mM sodium phosphate, pH 7.2-7.6) was applied at a volume ten times the weight of the HIC effluent concentrate. Inlet pressure was maintained at <40 psi, and outlet pressure was monitored. After all of the diafiltration buffer had been added, the sodium concentration of the permeate was determined. Diafiltration was considered complete if the sodium concentration of the permeate was within 10% of that of the diafiltration buffer. Additional diafiltration buffer (5× the weight of the concentrate) was added, and diafiltration extended, if necessary, until the sodium concentration of the permeate was within ±0% of that of the diafiltration buffer.

[0112] Following diafiltration, the ultrafiltration was continued until the concentrate had a mass of approximately 6 kg. Product was then gently blown out of the UF system (≦25 psi). The ultrafiltration unit was postwashed twice with 1.5 kg diafiltration buffer. The UF postwashes were added to the diafiltered concentrate. The total weight of concentrate was determined and the protein concentration determined (OD at 280 nm).

[0113] Based on the OD protein observed, the AAT protein concentration was determined, and adjusted if necessary to the range 2.9-6.8%. Analysis for LAL, SDS-PAGE, Bradford protein, potency, and bioburden were performed. SDS-PAGE showed ≧98% AAT. Based on AAT activity, the concentrates contained a total of 2096 g of AAT, a 79% yield of the activity present in the Cohn paste suspension, and a purification factor of 26.6. Specific activity for each of the three batches ranged from 0.886 to 1.04, and averaged 0.974 mg functional AAT per mg protein.

[0114] The AAT concentrate (2.9-6.8% protein) was adjusted to 20-25° C., and sucrose (1.75 kg per kg AAT concentrate) and potassium acetate (0.175 kg per kg AAT concentrate) were added. The final concentration of sucrose was 59.8%+6% (w/w), and the final concentration of potassium acetate was 5.98%±0.6% (w/w). After mixing, the stabilized concentrate was transferred into one-liter sealed serum bottles. The bottles were stored at 2-8° C. for no more than 10 weeks (and at 15-25° C. for no more than 48 hours) before being heat-treated (pasteurized). Pasteurization at 60±1° C. was performed for 10-11 hours. The pasteurized AAT solution was held at 2-8° C. for no more than 10 weeks, and at 15-25° C. for no more than 72 hours, prior to further processing.

[0115] Pasteurized AAT solution was pooled under HEPA-filtered air into two batches, and diluted with diafiltration buffer (20 mM sodium phosphate, 45 mM NaCl, 3% mannitol, pH 6.6-7.4) at a ratio of 5:1 buffer:AAT solution (w/w). The diluted solutions were sampled for LAL, protein, and potency. Based on AAT activity, the pasteurized and diluted solutions contained a total of 1941 g of AAT, a 73% yield of the activity present in the Cohn paste suspension, and a purification factor of 26.6. Specific activities for the two pasteurized batches were 0.954 and 0.993, an average of 0.973 mg functional AAT per mg protein. The percent monomer of the AAT solutions was measured by size-exclusion HPLC before and after pasteurization. The monomer fractions of the AAT concentrates (pre-pasteurization) were 97.1% to 98.5%, averaging 97.7%. The monomer fractions of the two pasteurized and diluted solutions were 95.9% and 97.5%, an average of 96.7%. Only 1.0% of the monomeric form of AAT was polymerized or aggregated during the pasteurization step.

[0116] Two YM100 filter cartridges (Millipore, Bedford, Mass.) were installed in series into a YM100 UF system, with the first cartridge operated in a tangential flow mode and the second cartridge dead-ended. The UF system was recirculated with a minimum of 5 kg diafiltration buffer. Following recirculation, the diafiltration buffer was tested to verify pH (6.8-7.2) and LAL (<0.25 EU/ml). The diafiltration buffer, and all subsequent processing until lyophilization, was at 2-8° C.

[0117] Each of the pooled AAT solutions was passed through the YM100 cartridges at 2-8° C. at an inlet pressure of ≦45 psi. The load did not exceed 1339 grams protein, and the weight of the YM100 filtrate plus postwashes did not

exceed 337 kg. The YM100 filtrates were then ultrafiltered and diafiltered, at an inlet pressure of ≦50 psi, against diafiltration buffer (1.60-1.90 mg/ml sodium, 10 times the YM100 concentrate weight), using an ultrafilter containing a 10,000 M.W. membrane (≧25 $ft^2$ surface area) that was dedicated to the post-pasteurization process.

[0118] The diafiltered solutions were sampled inline and tested for sodium. If the sodium level of the permeate was within ±10% of the diafiltration buffer sodium concentration, diafiltration was considered complete. If the sodium level was not within ±10% of the diafiltration buffer sodium concentration, diafiltration was repeated with additional diafiltration buffer (5 times the YM100 filtrate weight).

[0119] A final concentration was performed until approximately 6 kg of solution was obtained. Two postwashes were performed using 1.5 kg diafiltration buffer each time. Postwashes were combined with the concentrate for determination of total volume of diafiltered YM100 filtrate. Diafiltered YM100 filtrates were held for no more than 12 days at 2-8° C. before further processing. Based on AAT activity, the diafiltrate contained a total of 1960 g of AAT, a 74% yield of the activity present in the Cohn paste suspension, with a purification factor of 27.5. Specific activities for the two batches were 0.984 and 1.03, an average of 1.01 mg functional AAT per mg protein.

[0120] After addition of diafiltration buffer to obtain a final formulation target of 50 mg functional AAT/ml, the YM100 filtrate solution pH was adjusted as necessary to pH 6.8-7.2. Clarification was carried out with a 0.2 micron Pall SLK-7002-NRP Filter (Pall Corp., East Hills, N.Y.). Once clarified, the non-sterile bulk AAT solutions were combined, weighed and sampled for LAL, protein, potency, and bioburden (<100 CFU/ml). The non-sterile bulk AAT was held for no longer than 73.5 hours at 2-8° C. pending sterile filtration. Based on AAT activity, the non-sterile bulk AAT solution contained a total of 1822 g of AAT, a 69% yield of the activity present in the Cohn paste suspension, with a purification factor of 26.8. The specific activity was 0.981 mg functional AAT per mg protein.

[0121] In preparation for sterile filtration, a sterile bulk assembly consisting of a 60 L bulk receiver, a Pall 0.2 micron KA1NFP2 sterilizing filter and two (2) Millipore 0.2 Micron Aervent™ 50 vent filters was prepared. The assembly was autoclaved and used within 7 days of autoclaving. The non-sterile bulk solution was sterile-filtered with control of temperature (2-8° C.), pressure (≦20 psi), filtration time (≦120 minutes), and load including postwash (≦0.26 kg non-sterile bulk per $cm^2$ filter area). The sterile filtrate ultimately obtained from 667 kg of Cohn fraction IV paste contained 1.78 kg of functional AAT, corresponding to an overall yield of 67% based on the activity of the initial Cohn fraction $IV_{1-4}$ suspension, and a purification factor of 29.8. The specific activity was 1.09 mg functional AAT per mg protein. The product was >99% AAT by SDS-PAGE, and >95% monomer by size-exclusion HPLC.

[0122] AAT sterile bulk was aseptically filled into 50 ml Type I glass vials using a fill volume targeted to achieve approximately 1000 mg functional AAT activity per vial (i.e. 20.8 g±0.2 g solution per vial), and the vial contents were frozen and lyophilized.

TABLE 4

| | Fr. $IV_{1-4}$ Extract | Post-Aerosil | Filtrate | IEC Eluate | HIC Effluent | DF HIC Conc. | Diluted, Pasteur. | YM100 Filtrate | Non-Sterile Bulk | Final Container |
|---|---|---|---|---|---|---|---|---|---|---|
| No. of Batches | 9 | 19 | 9 | 9 | 3 | 3 | 2 | 2 | 1 | 1 |
| Yield (g AAT; total for all batches) | 2658 | 1833* | 1557* | 2241 | 2090 | 2096 | 1941 | 1960 | 1822 | 1780 |
| Overall Yield from Extract | 100% | 69% | 59% | 84% | 79% | 79% | 73% | 74% | 69% | 67% |
| Purification Factor | 1.0 | 1.4 | 1.5 | 16.2 | 25.6 | 26.6 | 26.6 | 27.5 | 26.8 | 29.8 |
| Specific Activity** (mg/mg) | 0.037† | 0.053† | 0.056† | 0.592† | 0.937‡ | .0974‡ | 0.973‡ | 1.01‡ | 0.981‡ | 1.09‡ |

*The AAT assay for these fractions is believed to be low, due to unidentified interfering factors.

**Specific activities are averages over the number of batches shown.

†The Bradford Protein assay was used for these fractions because they are too impure to determine protein concentration by $OD_{280}$. The protein standard used in the Bradford assay was purified AAT, calibrated using an extinction coefficient for AAT of 5.3, see R. Pannell, D. Johnson, and J. Travis, Biochemistry 13: 5439-5445 (1974).

‡Protein concentration by $OD_{280}$ using an extinction coefficient for AAT of 5.3.

[0123] Functional AAT yields, and characteristics of the AAT fractions obtained, at each of the above steps are set out in Table 4. Modifications of the above-described modes for carrying out the invention will be obvious to those of skill in the fields of protein purification, analytical chemistry, medicine, and related fields, and such substitutions and modifications are contemplated to be within the scope of the invention. The detailed embodiments described above are provided by way of example only, and are not intended to limit the scope of the following claims.

We claim:

1. A method for partially purifying AAT from an AAT-containing protein mixture, consisting essentially of:

(a) suspending the AAT-containing protein mixture in a buffer under conditions that permit the AAT to be dissolved;

(b) contacting the resulting suspension with a disulfide-reducing agent to produce a reduced suspension;

(c) contacting the reduced suspension with an insoluble protein-adsorbing material; and

(d) removing insoluble materials from the suspension.

2. A method for purifying AAT from a crude AAT-containing protein precipitate, comprising the steps of:

(a) suspending a crude AAT-containing protein precipitate in a buffer under conditions that permit the AAT to be dissolved;

(b) contacting the resulting suspension with a disulfide-reducing agent to produce a reduced suspension;

(c) without addition of a substantial amount of additional salts, contacting the reduced suspension with an insoluble protein-adsorbing material; and

(d) removing insoluble materials from the suspension.

3. The method of claim 1, wherein the crude AAT-containing protein precipitate is derived from serum.

4. The method of claim 2, wherein the crude AAT-containing protein precipitate is derived from serum.

5. The method of claim 3, wherein the crude AAT-containing protein precipitate is Cohn fraction $IV_{1}$ precipitate.

6. The method of claim 4, wherein the crude AAT-containing protein precipitate is Cohn fraction $IV_{1-4}$ precipitate.

7. The method of any of claims 1-6, wherein the disulfide-reducing agent is a dithiol.

8. The method of claim 7, wherein the dithiol is dithiothreitol.

9. The method of any of claims 1-6, wherein the protein-adsorbing material is a silica adsorbent.

10. The method of any of claims 1-6 wherein the protein-adsorbing material is fumed silica.

11. The method of claim 7, wherein the protein-adsorbing material is fumed silica.

12. The method of claim 8, wherein the protein-adsorbing material is fumed silica.

13. The method of claim 2, further comprising an anion exchange chromatography step.

14. The method of claim 3, further comprising an anion exchange chromatography step.

15. The method of claim 13, further comprising a hydrophobic interaction chromatography step.

16. The method of claim 14, further comprising a hydrophobic interaction chromatography step.

17. The method of claim 15, further comprising a viral reduction step.

18. The method of claim 16, further comprising a viral reduction step.

19. The method of claim 17, wherein the viral reduction step comprises pasteurization at about 60° C.

20. The method of claim 18, wherein the viral reduction step comprises pasteurization at about 60° C.

21. The method of claim 20, wherein the pasteurization step is carried out on a solution of AAT containing at least 40% w/w sucrose.

22. The method of claim 21, wherein the concentration of sucrose is at least 50%.

23. The method of claim 22, wherein the concentration of sucrose is about 60%.

24. The method of claim 19, wherein the viral reduction step further comprises filtration effective to remove viral particles.

25. The method of claim 23, wherein the viral reduction step further comprises filtration effective to remove viral particles.

26. The method of any one of claims 15-25, further comprising a sterilization step.

27. The method of claim 26, wherein the sterilization step comprises filtration effective to remove bacteria.

28. A method for purifying AAT from a crude AAT-containing protein precipitate, comprising the steps of:

(a) suspending an AAT-containing Cohn fraction $IV_{1-4}$ precipitate in a buffer under conditions that permit the AAT to be dissolved;

(b) contacting the resulting suspension with dithiothreitol to produce a reduced suspension;

(c) without addition of a substantial amount of additional salts, contacting the reduced suspension with fumed silica;

(d) removing insoluble materials from the suspension to obtain a clarified protein solution;

(e) anion exchange chromatography of the clarified protein solution;

(f) hydrophobic interaction chromatography of the product of the anion exchange chromatography;

(g) pasteurization of the product of the hydrophobic interaction chromatography at about 60° C.;

(h) filtration of the pasteurized product effective to remove viral particles; and

(i) sterile filtration.

29. An alpha-1-antitrypsin composition, containing:

(a) less than 6% contaminating proteins by SDS-PAGE;

(b) less than 0.1% albumin;

(c) less than 0.8% $\alpha_1$-acid glycoprotein;

(d) less than 0.1% $\alpha_2$-macroglobulin;

(e) less than 0.1% apolipoprotein A1;

(f) less than 0.5% antithrombin III;

(g) less than 0.1% ceruloplasmin;

(h) less than 0.5% haptoglobin;

(i) less than 0.2% IgA;

(j) less than 0.1% IgG; and

(k) less than 0.1%. transferrin;

wherein the specific activity of the alpha-1-antitrypsin is at least 0.99 mg functional AAT per milligram, when using as an extinction coefficient $E_{1\ cm,280\ nm}^{1\%}$=5.3;

wherein the apparent ratio of active to antigenic AAT is greater than 1.08, when measured by endpoint nephelometry;

wherein less than 8% of the composition is of a higher molecular weight than monomeric AAT; and

wherein the composition is stable for at least 2 years when stored lyophilized at up to 25° C.

30. The composition of claim 29, wherein the number of enveloped viruses are reduced by at least 11 $\log_{10}$ units and non-enveloped viruses are reduced by at least 6 $\log_{10}$ units.

31. The composition of claim 29 or claim 30, wherein the composition contains less than 2% contaminating proteins by SDS-PAGE, and wherein the apparent ratio of active to antigenic AAT is greater than 1.16.

32. The composition of claim 31, wherein the composition contains

(a) less than 1% contaminating proteins by SDS-PAGE;

(b) less than 0.2% $\alpha_1$-acid glycoprotein;

(c) less than 0.1% antithrombin III;

(d) less than 0.1% haptoglobin; and

(e) less than 0.1% IgA;

and wherein less than 5% of the composition is of a higher molecular weight than monomeric AAT, and the apparent ratio of active to antigenic AAT is greater than 1.23.

* * * * *

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| (51) International Patent Classification 6 : C07K 1/16, 1/18, 1/30, 14/81 | A1 | (11) International Publication Number: WO 95/35306 |
|---|---|---|
| | | (43) International Publication Date: 28 December 1995 (28.12.95) |

(21) International Application Number: PCT/US95/07616

(22) International Filing Date: 16 June 1995 (16.06.95)

(30) Priority Data:
08/261,406 17 June 1994 (17.06.94) US

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(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
*With international search report.*

(54) Title: PROCESS FOR SEPARATING ALPHA$_1$-PROTEINASE INHIBITOR FROM COHN FRACTION IV$_1$ AND IV$_4$ PASTE

(57) Abstract

The present invention is directed to a process for purifying $\alpha_1$-proteinase inhibitor. The process comprises providing an impure protein fraction which comprises $\alpha_1$-proteinase inhibitor. The impure protein fraction is precipitated with a precipitant comprising PEG. The supernatant from the PEG precipitation, which comprises $\alpha_1$-proteinase inhibitor, is collected and applied to an anion-exchange medium. A fraction comprising $\alpha_1$-proteinase inhibitor is recovered from the anion-exchange medium and applied to a metal chelate medium. A fraction comprising $\alpha_1$-proteinase inhibitor is then recovered from the metal chelate medium. Alpha$_1$-proteinase inhibitor purified by the process has a specific activity greater than 0.6 units/mg.

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# PROCESS FOR SEPARATING ALPHA$_1$-PROTEINASE INHIBITOR FROM COHN FRACTION IV$_1$ AND IV$_4$ PASTE

## Field of the Invention

The present invention relates to the purification of a serine proteinase inhibitor, $\alpha_1$-proteinase inhibitor.

## Background of the Invention

Alpha$_1$-Proteinase Inhibitor ($\alpha_1$-PI), also known as $\alpha_1$-antitrypsin, is a serum glycoprotein with a molecular weight of 52,000. Alpha$_1$-PI is synthesized in the liver and is present in the serum at levels between 150 and 350 mg/dl (equivalent to 30-80 $\mu$M) when assayed with plasma standards.

Alpha$_1$-PI functions in the lungs to inhibit neutrophil elastase, a serine protease, which in large quantities can lead to the destruction of the alveolar walls. In the normal lung, $\alpha_1$-PI provides more than 90% of the anti-neutrophil elastase protection in the lower respiratory tract.

Alpha$_1$-PI deficiency is an autosomal, recessive hereditary disorder displayed by a large number of allelic variants and has been characterized into an allelic arrangement designated as the protease inhibitor (Pi) system. These alleles have been grouped on the basis of the $\alpha_1$-PI levels that occur in the serum of different individuals. Normal individuals have normal serum levels of $\alpha_1$-PI (normal individuals have been designated as having a PiMM phenotype). Deficient individuals have

serum $\alpha_1$-PI levels of less than 35% of the average normal level (these individuals have been designated as having a PiZZ phenotype). Null individuals have undetectable $\alpha_1$-PI protein in their serum (these individuals have been designated as having a Pi(null)(null) phenotype).

Alpha$_1$-PI deficiency is characterized by low serum (less than 35% of average normal levels) and lung levels of $\alpha_1$-PI. These deficient individuals have a high risk of developing panacinar emphysema. This emphysema predominates in individuals who exhibit PiZZ, PiZ(null) and Pi(null)(null) phenotypes. Symptoms of the condition usually manifests in afflicted individuals in the third to fourth decades of life.

The emphysema associated with $\alpha_1$-PI deficiency develops as a result of insufficient $\alpha_1$-PI concentrations in the lower respiratory tract to inhibit neutrophil elastase, leading to destruction of the connective tissue framework of the lung parenchyma. Individuals with $\alpha_1$-PI deficiency have little protection against the neutrophil elastase released by the neutrophils in their lower respiratory tract. This imbalance of protease:protease inhibitor in $\alpha_1$-PI deficient individuals results in chronic damage to, and ultimately destruction of the lung parenchyma and alveolar walls.

Individuals with severe $\alpha_1$-PI deficiency typically exhibit endogenous serum $\alpha_1$-PI levels of less than 50 mg/dl, as determined by commercial standards. Individuals with these low serum $\alpha_1$-PI levels have greater than an 80% risk of developing emphysema over a lifetime. It is estimated that at least 40,000 patients in the United States, or 2% of all those with emphysema, have this disease resulting from a defect in the gene coding for $\alpha_1$-PI. A deficiency in $\alpha_1$-PI represents one of the most common lethal hereditary disorders of Caucasians in the United States and Europe.

Therapy for patients with $\alpha_1$-PI deficiency is directed towards replacement or augmentation of $\alpha_1$-PI

levels in the serum. If serum levels of $\alpha_1$-PI are increased, this is expected to lead to higher concentrations in the lungs and thus correct the neutrophil elastase:$\alpha_1$-PI imbalance in the lungs and prevent or slow destruction of lung tissue. Studies of normal and $\alpha_1$-PI deficient populations have suggested that the minimum protective serum $\alpha_1$-PI levels are 80 mg/dl or 11 $\mu$M (about 57 mg/dl; using pure standards). Consequently, most augmentation therapy in $\alpha_1$-PI deficient patients is aimed toward providing the minimum protective serum level of $\alpha_1$-PI, since serum $\alpha_1$-PI is the source of alveolar $\alpha_1$-PI.

Alpha$_1$-PI preparations have been available for therapeutic use since the mid 1980's. The major use has been augmentation (replacement) therapy for congenital $\alpha_1$-PI deficiency. The half-live of human $\alpha_1$-PI *in vivo* is 4.38 days with a standard deviation of 1.27 days. The currently recommended dosage of 60 mg $\alpha_1$-PI/kg body weight weekly will restore low serum levels of $\alpha_1$-PI to levels above the protective threshold level of 11 $\mu$M or 80 mg/dl.

Previously $\alpha_1$-PI has been purified by various techniques. One such process combined chromatography on an anion-exchange chromatography medium followed by PEG precipitation. Other purification procedures have used PEG precipitation followed by anion-exchange chromatography and others have used multiple PEG precipitation steps followed by anion-exchange chromatography. Still other methods have used phase separation techniques to purify $\alpha_1$-PI. Specific activities of 1.26 units/mg have been reported for purified $\alpha_1$-PI.

## Summary of the Invention

The present invention is directed to a process for purifying $\alpha_1$-proteinase inhibitor. The process comprises providing an impure protein fraction which comprises $\alpha_1$-proteinase inhibitor. The impure protein fraction is

precipitated with a precipitant comprising PEG. In a preferred embodiment the precipitant further comprises $ZnCl_2$. The supernatant from the PEG precipitation, which comprises $\alpha_1$-proteinase inhibitor is collected and applied to an anion-exchange medium. A fraction comprising $\alpha_1$-proteinase inhibitor is recovered from the anion-exchange medium and applied to a metal chelate medium. A fraction comprising $\alpha_1$-proteinase inhibitor is then recovered from the metal chelate medium. In a preferred embodiment the fraction comprising $\alpha_1$-proteinase inhibitor recovered from the metal chelate medium is further purified by chromatography on a second ion-exchange medium.

Alpha$_1$-proteinase inhibitor purified by the process has a specific activity greater than 0.6 units/mg.

## Detailed Description

The present invention describes a purification process for the purification of $\alpha_1$-PI. This purification procedure uses a unique combination of known purification steps to produce a high specific activity $\alpha_1$-PI preparation.

### Alpha$_1$-Proteinase Inhibitor Purification

Alpha$_1$-PI is purified from an impure protein fraction. The impure protein fraction may be plasma, $\alpha_1$-PI produced by recombinant methods or any other source comprising $\alpha_1$-PI protein. In a preferred embodiment $\alpha_1$-PI is prepared from frozen plasma. The plasma is thawed and the Cohn $IV_1$+$IV_4$ fraction is prepared. The preparation of the Cohn $IV_1$+$IV_4$ fraction (the Cohn $IV_1$+$IV_4$ precipitate) is well known in the art and is described briefly here.

### Preparation of $IV_1$+$IV_4$ Fraction

Plasma is maintained at a temperature of 1.5°C ± 1.5°C and the pH is adjusted to 7 ± 0.2 with either sodium bicarbonate or acetate buffer, pH 4.0. Sufficient cold SD3A ethanol (95% v/v ethanol and 5% v/v methanol) is added to bring the plasma to a final alcohol concentration of 8% v/v. During the alcohol addition the temperature of the plasma is lowered to from -2°C ± 1°C. The precipitate which forms is removed by centrifugation in a Sharples or Westphalia centrifuge or by filtration through a filter press, at -2°C ± 1°C. The result precipitate and supernatant are designated the Fraction I precipitate and supernatant.

The Fraction I supernatant is adjusted to pH 6.9 ± 0.1 by the addition of pH 4 acetate buffer (0.8 M sodium acetate adjusted to pH 4 with acetic acid) and is brought to 20% v/v alcohol by the addition of cold SD3A alcohol. During the alcohol addition the temperature is lowered to -5.5°C ± 1.5°C. The precipitate which forms is removed by centrifugation in a Sharples or Westphalia centrifuge or

by filtration through a filter press, at -5.5°C ± 1.5°C. The result precipitate and supernatant are designated the Fraction II+III precipitate and supernatant.

If required, the Fraction II+III supernatant is filtered through a 5 to 30 micron filter to remove particulate matter.

In one embodiment of the present invention, Antithrombin III (AT-III) Poor Fraction II and III is prepared as follows.

Heparin immobilized medium is equilibrated with 10 mM ± 5 mM sodium citrate, pH 6.5-7.5 and then 10 mM ± 5 mM sodium citrate, pH 6.5-7.5, 150 mM ± 50 mM NaCl, 20% w/v SD3A alcohol. The medium is equilibrated in a -4°C to -7°C environment until the effluent is -4°C to -7°C.

The Fraction II+III supernatant is passed through the heparin immobilized medium packed in a column. The medium adsorbed AT-III is washed with 10 mM ± 5 mM sodium citrate, 150 mM ± 50 mM NaCl, 2% w/v SD3A alcohol pH 6.5-7.5. The AT-III-poor effluent and the wash effluent are pooled and processed further.

Alternatively, the plasma suspension containing 8% v/v alcohol, at -2°C ± 1°C, pH 7 ± 0.2 described above is adjusted to pH 6.9 ± 0.1 by the addition of pH 4 acetate buffer, and is then processed further without the removal of the precipitate. The alcohol concentration is raised to 20% v/v by the addition of cold SD3A alcohol and the temperature is gradually lowered to -5.5°C ± 1.5°C. The precipitate which forms is removed by centrifugation in a Sharples or Westphalia centrifuge or by filtration through a filter press, at -5.5°C ± 1.5°C. The resultant precipitate and supernatant are designated the Fraction I+II+III precipitate and supernatant.

The Fraction II+III, the Fraction II+III, AT-III poor and/or the Fraction I+II+III supernatant is/are maintained at -5.5°C ± 1.5°C and the pH is adjusted to 5.2 ± 0.1 by the addition of pH 4 acetate buffer.

The resultant suspension is allowed to settle for at least 6 hours at -5.5°C ± 1.5°C, after which time the pH is adjusted to 5.8 ± 0.1 with either sodium acetate, pH 4.0 or sodium bicarbonate buffer, pH 4.0. The alcohol concentration is adjusted to 40% v/v by the addition of cold SD3A alcohol. The precipitate which forms is removed by centrifugation in a Sharples or Westphalia centrifuge or by filtration through a filter press, at -5.5°C ± 1.5°C. The result precipitate and supernatant are designated the Fraction $IV_1+IV_4$ precipitate and supernatant. The Fraction $IV_1+IV_4$ precipitate is further purified for production of $\alpha_1$-PI.

The Fraction $IV_1+IV_4$ precipitate may be frozen until processed further or until sufficient material has been accumulated for further processing.

PEG/$ZnCl_2$ Precipitation

The $IV_1+IV_4$ precipitate is resuspended in water for injection (WFI), in a ratio of about 3 to 10 parts of water per part of $IV_1+IV_4$ precipitate, at about 0° to 10°C and the pH is adjusted to 8.5 ± 0.5 (the Water Extract). After the precipitate is resuspended solid Tris is added to a final concentration of 10 ± 5 mM and NaCl (5 ± 0.5 M) is added to a final concentration of 150 ± 20 mM. Polyethylene glycol 3350 (PEG) and $ZnCl_2$ are added to a final concentration of 15 ± 7.5% w/w PEG and 0.5 ± 0.25 mM $ZnCl_2$. The suspension is adjusted to pH 8 ± 1 and mixed for about one hour.

The PEG/$ZnCl_2$ precipitate which forms is removed by passing the suspension through a filter press at 0°C-10°C. The filter press is washed before and after filtering with 150 ± 25 mM NaCl, 15 ± 7.5% w/w PEG and 5 ± 5 mM $ZnCl_2$, pH 8 ± 1. Alternatively, the precipitate may be removed by centrifugation at about 6,000 rpm for 10-15 minutes.

## $ZnCl_2$ Precipitation

$ZnCl_2$ (100 ± 10 mM) is added to the supernatant (the 15%PEG-$ZnCl_2$ supernatant) to a final concentration of 10 ± 5 mM and the solution is adjusted to pH 8 ± 1. The solution is mixed for about one hour. The $ZnCl_2$ precipitate which forms is recovered by centrifugation, filter press, or other suitable method of recovery. The precipitate may be frozen for future processing.

For further processing the $ZnCl_2$ precipitate (the 10 mM $ZnCl_2$ precipitate) is re-solubilized in about 50 mM EDTA and adjusted to a conductivity of not more than 5 mS and to a pH of 8 ± 1.

## Anion-Exchange Chromatography

The re-solubilized $ZnCl_2$ precipitate is then applied to diethyl(2-hydroxpropyl)aminoethyl (QAE) chromatography medium or other similar anion-exchange medium. Either batch or column chromatography may be used. The medium is equilibrated at 0°-10°C with cold water for injection (CWFI), prior to absorption of $\alpha_1$-PI to the chromatography medium. After $\alpha_1$-PI has been absorbed onto the medium it is washed with 50 ± 25 mM NaCl, 10 ± 5 mM sodium phosphate, pH 8 ± 1 to remove unbound material. $Alpha_1$-PI is then eluted from the anion-exchange chromatography medium with 150 ± 50 mM NaCl, 10 ± 5 mM sodium phosphate, pH 8 ± 1. The eluate which includes $\alpha_1$-PI (the 1st QAE Eluate) is collected for further processing.

After the removal of $\alpha_1$-PI, the anion-exchange medium is cleaned by washing with, in sequence: 2 ± 0.2 M NaCl, 10 ± 5 mM sodium phosphate, pH 8 ± 1; WFI or 500 mM NaOH; WFI. The chromatography medium is then stored in either 2 ± 0.2 M NaCl, 10 ± 5 mM sodium phosphate, pH 8 ± 1 or 50 mM NaOH until required.

SD Treatment

The anion-exchange medium eluate is concentrated/diafiltered by ultrafiltration against 150 ± 25 mM NaCl, 50 ± 10 mM sodium phosphate, 1 ± 0.1 mM imidazole, pH 7.5 ± 1 to concentrate the $\alpha_1$-PI and to remove EDTA which co-elutes from the anion-exchange chromatography medium with the $\alpha_1$-PI, to form the 10K UF.

A solution of 10 ± 1% w/v polysorbital 80 and 3 ± 0.3% w/v tri-n-butyl phosphate is added to the diafiltered $\alpha_1$-PI to a final concentration of 1 ± 0.5% w/v polysorbital 80 and 0.3 ± 0.15% w/v tri-n-butyl phosphate. The solution is then incubated at 27° ± 3°C, pH 8 ± 1 for 6.5 ± 0.5 hours to inactivate any viruses which may be present in the $\alpha_1$-PI. After the incubation the treated $\alpha_1$-PI solution is cooled to 0°-10°C and, if necessary, the pH is adjusted to 7.5 ± 1. In other embodiments of the present invention the SD treatment is performed after ultrafiltration, as described below or the SD treatment may be performed at this step as well as at the step described below.

Metal Chelate Chromatography

The $\alpha_1$-PI is then applied to a copper, zinc or similar metal ion primed medium, such as MATREX-CELLUFINE CHELATE (supplied by Chisso of Japan), at 0°-10°C. Prior to use the medium is washed with, in sequence: WFI; 6 ± 0.6 mg/ml $CuSO_4.5H_2O$; WFI and 150 ± 25 mM NaCl, 250 ± 25 mM sodium acetate, pH 5 ± 1. The resin is then equilibrated with 150 ± 25 mM NaCl, 50 ± 10 mM sodium phosphate, 1 ± 0.1 mM imidazole, pH 7.5 ± 1 at 0°-10°C. Either batch or column chromatography can be used. The SD treated fraction is applied to the metal chelate chromatography medium to absorb $\alpha_1$-PI to the metal chelate chromatography medium. The $\alpha_1$-PI absorbed medium is washed with 500 ± 50 mM NaCl, 50 ± 10 mM sodium phosphate, 1 ± 0.1 mM imidazole, pH 7.5 ± 1 to remove any unbound material from the chromatography medium. The $\alpha_1$-PI is eluted with 150

± 25 mM NaCl, 50 ± 10 mM sodium phosphate, 5 ± 2.5 mM imidazole, pH 7.5 ± 1. The $\alpha_1$-PI containing eluate (the $Cu^{++}$ Eluate) is collected and may be frozen until processed further.

The chromatography medium is cleaned with, and may be stored in, 500 ± 50 mM NaCl, 50 ± 25 mM EDTA, pH 7 ± 1 or the medium may be washed with CWFI and cleaned with 500 mM NaOH and stored in 50 mM NaOH.

Ultrafiltration

The $\alpha_1$-PI containing eluate is ultrafiltered using a high (100,000) molecular weight cut-off ultrafiltration membrane, to remove high molecular weight contaminants and any viral contaminants which may be present in the metal chelate medium eluate.

The filtrate is collected and concentrated/diafiltered by ultrafiltration against 50 ± 25 mM NaCl, 10 ± 5 mM sodium phosphate, pH 8 ± 1, containing up to 20 mM EDTA, to form the 100K UF.

SD Treatment

A solution of 10 ± 1% w/v polysorbital 80 and 3 ± 0.3% w/v tri-n-butyl phosphate is added to the diafiltered $\alpha_1$-PI to a final concentration of 1 ± 0.5% w/v polysorbital 80 and 0.3 ± 0.15% w/v tri-n-butyl phosphate. The solution is then incubated at 27° ± 3°C, pH 8 ± 1 for 6.5 ± 0.5 hours to inactivate any viruses which may be present in the $\alpha_1$-PI. After the incubation the treated $\alpha_1$-PI solution is cooled to 0°-10°C and, if necessary, the pH is adjusted to 7.5 ± 1.

Anion-Exchange Chromatography

The concentrated $\alpha_1$-PI is then applied to QAE chromatography medium or other similar anion-exchange medium, equilibrated at 0°-10°C with CWFI, as described above. The chromatograph medium is then washed with 50 ± 25 mM NaCl, 10 ± 5 mM sodium phosphate, pH 8 ± 1. Alpha$_1$-

PI is eluted from the anion-exchange medium with 150 ± 50 mM NaCl, 10 ± 5 mM sodium phosphate, pH 8 ± 1. The eluate (the 2nd QAE Eluate) is collected and its pH adjusted to 7.5 ± 1. The eluate may be frozen until processed further. If necessary the eluate is concentrated by ultrafiltration.

The $\alpha_1$-PI is filtered through a 5 micron filter to remove any particulate matter. The concentration of the $\alpha_1$-PI is adjusted to a desired level and the $\alpha_1$-PI is sterile filtered through a 0.22 micron filter, dispensed into vials and lyophilized (the 5$\mu$ Filtrate).

The lyophilized $\alpha_1$-PI is redissolved in sterile water for injection for administration to patients (the Final Container).

Alpha$_1$-PI is stored at 2-8°C.

## Alpha$_1$-PI Activity Assays

A chromogenic assay is used to detect $\alpha_1$-PI activity. The assay utilizes a trypsin sensitive chromogenic substrate which releases p-nitroaniline in the presence of trypsin (supplied by Sigma Chemical Co. of St Louis, Missouri). The p-nitroaniline released is detected at 405 nm. $\alpha_1$-PI inhibits the release of p-nitroaniline from the substrate. The activity of $\alpha_1$-PI in the product can be determined by reference to a standard $\alpha_1$-PI activity curve.

## Protein Content

Protein content is determined by a Bio-Rad® assay method utilizing differential color change of a Coomassie Blue dye in response to various concentrations of protein measured at 595 nm. The protein content is calculated from a standard curve.

Administration

Alpha$_1$-PI is infused into a patient at a rate of about 0.08 ml/kg body weight per minute for the first 10 minutes. If the patient does not experience any discomfort, the rate is increased as tolerated. If tolerated, subsequent infusions to the same patient may be at the higher rate. If adverse events occur, the rate should be reduced or the infusion interrupted until the symptoms subside. The infusion may then be resumed at a rate which is tolerated by the patient.

If large doses are to be administered, several reconstituted vials of $\alpha_1$-PI may be pooled in an empty, sterile I.V. infusion container using aseptic technique.

Example 1
Purification of Alpha$_1$-PI

Twenty kg of $IV_1$+$IV_4$ precipitate was resuspended in 180 kg of WFI at 3.8°C and the pH was adjusted to 8.94. After the precipitate was resuspended 242.3 g of Tris, 6.7 kg of 1 M NaCl, and 35.4 kg of PEG were added and the solution mixed for 60 minutes. Then 2.2 kg of 100 mM $ZnCl_2$ was added and the suspension was adjusted to pH 7.92 and mixed for an additional 60 minutes at 0-8°C.

The PEG/$ZnCl_2$ precipitate which formed was removed by passing the suspension through a filter press at 0-8°C after the addition of 977 g of filtra-Cell BH 20 filter Aid (supplied by Celite of Germany). The filter press was washed before and after filtering with 30 kg of 150 mM NaCl, 15% w/w PEG, 0.5 mM $ZnCl_2$, pH 8.0.

27.8 kg of 100 mM $ZnCl_2$ was added to the supernatant and the solution was adjusted to pH 8. The precipitate which formed in the presence of the $ZnCl_2$ was recovered by centrifugation in a Sharples centrifuge. The $ZnCl_2$ precipitate was re-solubilized in 20 kg of 50 mM EDTA and adjusted to a conductivity of 6.48 mS and to a pH of 7.97.

The re-solubilized $ZnCl_2$ precipitate was then applied to diethyl(2-hydroxpropyl)aminoethyl (QAE) chromatography

medium (supplied by Toso Haas) packed into a 20 l column with an internal diameter of 250 cm. The QAE medium was equilibrated at 4°C with CWFI. The $\alpha_1$-PI was then absorbed into the chromatography medium. The chromatograph medium was then washed with 60 l of 50 mM NaCl, 10 mM sodium phosphate, pH 7.92. Alpha$_1$-PI was eluted from the anion-exchange medium with 60 l of 150 mM NaCl, 10 mM sodium phosphate, pH 8.06. The flow rate of the column was maintained at 600 ml/minute. The $\alpha_1$-PI containing eluate was collected.

The anion-exchange medium eluate was concentrated/diafiltered by ultrafiltration in a Millipore PELLICON unit (supplied by Millipore of Bedford MA) against 150 mM NaCl, 50 mM sodium phosphate, 1 mM imidazole, pH 7.5 to concentrate the $\alpha_1$-PI and to remove EDTA which co-elutes with the $\alpha_1$-PI.

1.1 kg of a solution of 10% w/v polysorbital 80 and 3% w/v tri-n-butyl phosphate was added to the diafiltered $\alpha_1$-PI and the solution was incubated at 25°C for 1 hour to inactivate any viral contaminants present in the diafiltered $\alpha_1$-PI. The solution was then cooled to 4°C and the pH adjusted to 7.33.

The $\alpha_1$-PI was then applied to 10 l of MATREX CELLUFINE CHELATE, a copper chelating medium (supplied by Chisso of Japan) at 4°C. Prior to use the medium was washed with, in sequence: WFI; 6 mg/ml $CuSO_4.5H_2O$; WFI and 150 mM NaCl, 250 mM sodium acetate, pH 5. The column was then equilibrated with 150 mM NaCl, 50 mM sodium phosphate, 1 mM imidazole, pH 7.5 at 4°C. The $\alpha_1$-PI absorbed medium was washed with 100 l of 500 mM NaCl, 50 mM sodium phosphate, 1 mM imidazole, pH 7.52 to remove any unbound material from the medium. The $\alpha_1$-PI bound to the chromatography medium was eluted with 150 mM NaCl, 50 mM sodium phosphate, 5 mM imidazole, pH 7.47. The flow rate was maintained at about 550 ml/minute. The $\alpha_1$-PI containing eluate was collected.

The eluate was ultrafiltered using a 100K CENTRASETTE supplied by Filtron. The filtrate was collected and concentrated/diafiltered by ultrafiltration in a Millipore PELLICON filtration unit against 50 mM NaCl, 20 mM EDTA, 10 mM sodium phosphate, pH 7.9.

The concentrated $\alpha_1$-PI was again applied to 5 l of QAE chromatography medium, equilibrated at 0°-10°C with CWFI, to absorb $\alpha_1$-PI to the chromatography medium. The chromatograph medium was then washed with 24 l of 50 mM NaCl, 10 mM sodium phosphate, pH 8. $Alpha_1$-PI was eluted from the chromatography medium with 150 mM NaCl, 10 mM sodium phosphate, pH 8. The pH of the eluate was adjusted to 7.5. The eluate was concentrated/diafiltered by ultrafiltration in a Millipore PELLICON filtration unit against 50 mM NaCl, 10 mM sodium phosphate, pH 7.9.

Throughout the purification, aliquots of the $\alpha_1$-PI containing solutions were collected and analyzed. The results are summarized in Table I.

Table I

| Sample | $\alpha_1$-PI Activity (%) | $A_{280\,nm}$ (kg) | U $A_{280\,nm}$ (%) | Specific Activity (U/mg) |
|---|---|---|---|---|
| Water Extract | 3,460 (100) | 31.3 (5,000) | 156,500 (100) | 0.022 |
| 15%PEG-$ZnCl_2$ Supernatant | 2,478 (72) | 5.22 (5,078) | 26,507 (17) | 0.093 |
| 10 mM $ZnCl_2$ Precipitate | 2,322 (67) | 7.72 (3,600) | 27,792 (18) | 0.084 |
| 1st QAE Eluate | 1,612 (47) | 3.95 (3,008) | 11,882 (8) | 0.136 |
| 10K UF | 1,764 (51) | 19.5 (601) | 11,720 (7) | 0.151 |
| $Cu^{++}$ Eluate | 1,445 (42) | 1.08 (3,261) | 3,521 (2) | 0.443 |
| 100 K UF | 1,371 (40) | 0.59 (5,396) | 3,184 (2) | 0.431 |
| 10K UF | 1,406 (41) | 5.98 (503) | 3,007 (2) | 0.467 |
| 2nd QAE Eluate | 1,181 (34) | 2.13 (839) | 1,787 (1) | 0.661 |

The purification procedure produced a final $\alpha_1$-PI fraction with a specific activity of 0.661 U/mg and a yield of 34%.

## Example 2

The purification procedure described in Example 1 was repeated except the $\alpha_1$-PI was filtered through a 0.22 micron filter. The filtrate was then dispensed into sterile vials and lyophilized.

The results are summarized in Table II.

Table II

| Sample | $\alpha_1$-PI Activity (%) | $A_{280\,nm}$ (kg) | U $A_{280\,nm}$ (%) | Specific Activity (U/mg) |
|---|---|---|---|---|
| Water Extract | 75,800 (100) | 16.8 (200) | 3,360,000 (100) | 0.023 |
| 15%PEG-$ZnCl_2$ Supernatant | 45,713 (60) | 1.58 (249.8) | 394,684 (12) | 0.116 |
| 10 mM $ZnCl_2$ Precipitate | 30,995 (41) | 9.03 (33.4) | 301.602 (9) | 0.103 |
| 1st QAE Eluate | 36,762 (49) | 1.87 (60.2) | 112,574 (3) | 0.327 |
| 10K UF | 26,938 (49) | 11.34 (9.7) | 109,998 (3) | 0.336 |
| After S/D Treatment | 34,906 (46) | 11.81 (10.8) | 127,548 (4) | 0.274 |
| $Cu^{++}$ Eluate | 23,435 (31) | 0.76 (60.4) | 45,904 (1) | 0.510 |
| 100 K UF | 21,952 (29) | 0.45 (89.6) | 40,320 (1) | 0.545 |
| 10K UF | 21,859 (29) | 3.43 (10.99) | 37,696 (1) | 0.580 |
| 2nd QAE Eluate | 10,270 (25) | 1.14 (18.6) | 21,204 (1) | 0.909 |
| 10 K UF | 24,461 (32) | 10.99 (2.45) | 26,926 (1) | 0.909 |
| 5$\mu$ Filtration | 21,648 (29) | 10.94 (2.478) | 27,109 (1) | 0.799 |
| Final Container | 17,850 (24) | 11.02 (2.1) | 23,142 (1) | 0.773 |

The purification procedure produced a final $\alpha_1$-PI fraction with a specific activity of 0.773 U/mg and a yield of 24%.

## Example 3
## Stability of the Purified Alpha$_1$-PI

Final container samples of $\alpha_1$-PI were stored in temperature controlled incubators at 5°C. After three months, storage samples were analyzed and compared to samples analyzed prior to storage. After reconstitution, the samples were incubated at 20° C for 0, 2 or 4 hours prior to analysis. Results for storage at 5°C for 0 and 3 months are summarized in Table III.

Table III

| Test Description | Months of storage at 5°C 0 | 3 |
|---|---|---|
| $\alpha_1$-PI activity | 205 U/vial | 203 U/vial |
| $\alpha_1$-PI Activity after reconstitution: | | |
| 0 hours | 213 U/vial | 203 U/vial |
| 2 hours | 223 U/vial | 210 U/vial |
| 4 hours | 188 U/vial | 208 U/vial |
| Elastase inhibitory activity after reconstitution | | |
| 0 hours | 323 U/vial | 323 U/vial |
| 2 hours | 305 U/vial | 298 U/vial |
| 4 hours | 318 U/vial | 308 U/vial |
| Protein content | 0.440 g/vial | 0.453 g/vial |
| Physical | | |
| Appearance | Pass | Pass |
| Moisture | 0.50% w/w | 0.37% w/w |
| Solubility | 1 minute | 1 minute |
| Vacuum | Present | Present |

After 3 months of storage at 5°C, samples of $\alpha_1$-PI retained 99% of their original activity. At manufacture, $\alpha_1$-PI activity of samples at 0, 2 or 4 hours after reconstitution was 213, 223, and 188 U/vial, respectively. Following storage at 5°C for 3 months the $\alpha_1$-PI activity following reconstitution was 203, 210, and 208 U/vial at 0, 2 and 4 hours, respectively.

Elastase inhibitory activity was also measured following reconstitution of the samples. At the time of manufacture, elastase inhibitory activity at 0, 2 or 4 hours after reconstitution was 323, 305, and 318 U/vial, respectively. Following storage for 3 months at 5°C, the elastase inhibitory activity was 323, 298, and 308 U/vial at 0, 2 or 4 hours after reconstitution, respectively.

Moisture content of the $\alpha_1$-PI sample at manufacture was 0.50% and after 3 months of storage at 5°C it was 0.37%.

Further experiments have shown that alpha$_1$-PI remains stable for at least 9 months following storage at 5°C. Samples stored at 5°C retained 99% of their original $\alpha_1$-PI activity.

## Example 4
## Comparison of $\alpha_1$-PI and Commercially Available $\alpha_1$-PI

$\alpha_1$-PI prepared in Example 2 was analyzed and compared to commercially available $\alpha_1$-PI obtained from the Cutter Biological division of Miles, Inc. The protein composition of the samples were analyzed by radial immunodiffusion.

Table IV

| Protein | $\alpha_1$-PI prepared in Example 2 mg/ml (% Total) | Cutter 01J081 mg/ml (% Total) | Cutter 01K047 mg/ml (% Total) |
|---|---|---|---|
| *Major Proteins* | | | |
| $\alpha_1$-PI | 23.80 (95) | 29.19 (91) | 35.14 (91) |
| Haptoglobin | 1.13 (5) | 0.60 (2) | 0.68 (2) |
| Albumin | <0.50 | 1.53 (5) | 2.27 (6) |
| IgA | <0.01 | 0.92 (3) | 0.90 (2) |
| *Minor Proteins* | | | |
| α1-Antichymotrypsin | <0.171 | <0.171 | <0.171 |
| α2-Antiplasmin | 0.041 | 0.083 | 0.106 |
| α2-Macroglobulin | <0.50 | <0.50 | <0.50 |
| Antithrombin III | <0.060 | 0.192 | 0.35 |
| Apolipoprotein A1 | 0.06 | 0.21 | 0.17 |
| Apoliprotein B | <0.095 | <0.095 | <0.095 |
| C1-Inactivator | <0.045 | 0.091 | 0.101 |
| Ceruloplasmin | <0.100 | <0.100 | <0.100 |
| HMW Kininogen | 0.009 | <0.001 | <0.001 |
| IgG | <0.020 | <0.020 | <0.020 |
| Prealbumin | 0.05 | <0.05 | <0.05 |
| Protein-C | <0.00125 | <0.00125 | <0.00125 |
| Protein-S | <0.001 | <0.001 | <0.001 |
| Transferrin | <0.50 | <0.50 | <0.50 |
| % Total = Percent of the Major Immunologically-Detected Plasma Proteins | | | |

## Example 5
## *In Vivo* Use of Alpha$_1$-PI

A group of three rabbits was administered $\alpha_1$-PI intravenously over a period of approximately one minute at a dose of 240 mg/kg of body weight (4 times the clinical dose of 60 mg/kg of body weight). A control rabbit was injected with 2.73 ml/kg body weight of 750 mM NaCl, 50 mM sodium phosphate, pH 7.5, over a period of one minute. Clinical observations were recorded immediately after administration and again at 30 and 72 hours after administration. Body weights were recorded prior to

infusion and at the end of the infusion. A gross necropsy was performed on all animals at the completion of the study.

Clinical signs observed in the $\alpha_1$-PI-treated group included decreased activity and dyspnea. There was no apparent effect on mean body weight of the animals in any group during this study. None of the rabbits died in the $\alpha_1$-PI-treated groups when a dose equivalent to 240 mg $\alpha_1$-PI/kg of body weight (4 times the clinical dose of 60 mg/kg of body weight) was given. Furthermore, no visible lesions were observed in any of the animals at terminal necropsy.

Alpha$_1$-PI was non-toxic when administered intravenously at a dose of 240 mg/kg of body weight (4 times the clinical dose of 60 mg/kg of body weight).

## Example 6
## *In Vivo* Use of Alpha$_1$-PI

A group of three mice were administered $\alpha_1$-PI intravenously over a period of approximately one minute at a dose of 1500 mg/kg of body weight (25 times the clinical dose of 60 mg/kg of body weight). A group of three control mice were injected with 17.0 ml/kg of body weight, 750 mM NaCl, 50 mM sodium phosphate pH 7.5, over a period of one minute. Clinical observations were recorded immediately after dosing and again at 24, 48 and 72 hours. Body weights were recorded prior to the infusion and at the end of the infusion. A gross necropsy was performed on all animals at the completion of the study.

The only clinical sign observed was decreased activity. There was no apparent effect on mean body weight of the animals during this study. None of the mice died when a dose of equivalent to 1,500 mg $\alpha_1$-PI/kg of body weight (25 times the clinical dose of 60 mg/kg of body weight) was given. Furthermore, no visible lesions were observed in any of the animals at terminal necropsy.

Based upon the results from the acute intravenous toxicity study in mice, $\alpha_1$-PI was found to be non-toxic when administered intravenously at 1,500 mg/kg of body weight (25 times the clinical dose of 60 mg/kg of body weight).

## Example 7
## *In Vivo* Use of Alpha$_1$-PI

A rabbit study lasting 33 days was designed to evaluate the potential toxic effect(s) associated with repeated intravenous exposure to $\alpha_1$-PI. For this study, five consecutive daily injections at twice the anticipated clinical dose of 60 mg/kg of body weight were administered. Preliminary hematological, clinical, biochemical, and gross necropsy data obtained from animals at day 6 and day 33 after the fifth repeated intravenous infusion of $\alpha_1$-PI were obtained. Alpha$_1$-PI was prepared by reconstitution of lyophilized powder with 5 ml Sterile Water for Injection to a concentration of 88 mg $\alpha_1$-PI/ml. A 5X buffer (750 mM NaCl, 50 mM sodium phosphate, pH 7.5) containing a concentration of salt similar to that within the reconstituted test-article served as the control. Male and female Albino New Zealand White rabbits (2.0 to 3.0 kg) were used as the test and control recipients.

Twelve (12) rabbits were administered intravenous equivalent-volume injections of either a 5X buffer (6 animals) or $\alpha_1$-PI (6 animals) at a dose of 120 mg (1.4 ml)/kg. Infusions of the 5X buffer and $\alpha_1$-PI were repeated daily for five consecutive days. The animals were separated into two sex-matched groups of six animals, three received control solution and three received the $\alpha_1$-PI solution. Each group of six animals were evaluated at day 6 and day 33 after commencement of the infusions. Following each infusion, all rabbits were observed at 30 and 60 minutes, then hourly for four hours. After the last infusion, the animals were monitored daily for pharmacotoxic signs and mortality.

Repeated administration of $\alpha_1$-PI at 120 mg/kg of body weight (two-times the clinical dose of 60 mg/kg of body weight) or an equal volume of 5X buffer control for five consecutive days, resulted in no significant perturbations in hematologic, clinical or biochemical parameters among rabbits examined at day 6 or day 33 after administration of the final dose.

The present invention is not limited to the specific embodiment given. It will be obvious to one skilled in the art that variations, such as variations in buffer concentration and types of buffers and salts, could also be used. Therefore, the present invention is not intended to be limited to the working embodiments described above. The scope of the invention is defined in the following claims.

WHAT IS CLAIMED IS:

1. A process for purifying $\alpha_1$-proteinase inhibitor comprising:

providing an impure protein fraction comprising $\alpha_1$-proteinase inhibitor;

suspending the impure protein fraction comprising $\alpha_1$-proteinase inhibitor in water;

precipitating the impure protein fraction comprising $\alpha_1$-proteinase inhibitor with a precipitant comprising PEG;

collecting a supernatant from the PEG precipitation, wherein the supernatant comprises $\alpha_1$-proteinase inhibitor;

applying the supernatant from the PEG precipitation to an anion-exchange medium;

recovering a fraction comprising $\alpha_1$-proteinase inhibitor from the anion-exchange medium;

applying the fraction comprising $\alpha_1$-proteinase inhibitor recovered from the anion-exchange chromatography medium to a metal chelate medium; and

recovering a fraction comprising $\alpha_1$-proteinase inhibitor from the metal chelate medium.

2. A process as recited in claim 1 wherein the impure protein fraction comprising $\alpha_1$-proteinase inhibitor is suspended in 3 to 10 parts of water for each part of impure protein fraction comprising $\alpha_1$-proteinase inhibitor.

3. A process as recited in claim 1 wherein the PEG precipitant further comprises $ZnCl_2$.

4. A process as recited in claim 1 wherein the PEG is present at a concentration of 7.5% to 22.5% w/w.

5. A process as recited in claim 3 wherein the $ZnCl_2$ is present at a concentration of 0.25 to 0.75 mM.

6. A process as recited in claim 1 further comprising precipitating the PEG precipitated fraction which comprises $\alpha_1$-proteinase inhibitor with $ZnCl_2$.

7. A process as recited in claim 6 wherein the $ZnCl_2$ is present at a concentration of 5 to 15 mM.

8. A process as recited in claim 1 further comprising applying the fraction comprising $\alpha_1$-proteinase inhibitor recovered from the metal chelate chromatography medium to a second anion-exchange medium.

9. A process as recited in claim 1 further comprising treating the fraction comprising $\alpha_1$-proteinase inhibitor recovered from the anion-exchange medium to inactivate any viral contaminants present in the fraction comprising $\alpha_1$-proteinase inhibitor.

10. A process as recited in claim 1 further comprising treating the fraction comprising $\alpha_1$-proteinase inhibitor recovered from the metal chelate medium to inactivate any viral contaminants present in the fraction comprising $\alpha_1$-proteinase inhibitor.

11. A process as recited in claim 9 wherein the fraction comprising $\alpha_1$-proteinase inhibitor is treated with solvent and detergent.

12. A process as recited in claim 9 wherein the fraction comprising $\alpha_1$-proteinase inhibitor is treated with tri-n-butyl phosphate and polysorbate 80.

13. A process as recited in claim 9 wherein the fraction comprising $\alpha_1$-proteinase inhibitor is treated with 0.15 to 0.45% w/v tri-n-butyl phosphate and 0.5 to 1.5% w/v polysorbate 80.

14. A process as recited in claim 10 wherein the fraction comprising $\alpha_1$-proteinase inhibitor is treated with solvent and detergent.

15. A process as recited in claim 10 wherein the fraction comprising $\alpha_1$-proteinase inhibitor is treated with tri-n-butyl phosphate and polysorbate 80.

16. A process as recited in claim 10 wherein the fraction comprising $\alpha_1$-proteinase inhibitor is treated with 0.15 to 0.45% w/v tri-n-butyl phosphate and 0.5 to 1.5% w/v polysorbate 80.

17. A process as recited in claim 1 wherein the metal chelate medium is selected from the group consisting of $Cu^{++}$ chelate medium, $Zn^{++}$ chelate medium.

18. A process as recited in claim 16 wherein the fraction comprising $\alpha_1$-proteinase inhibitor is eluted from the metal chelate medium with an aqueous solution comprising imidazole.

19. A process as recited in claim 16 wherein the fraction comprising $\alpha_1$-proteinase inhibitor is eluted from the metal chelate medium with an aqueous solution comprising 2.5 to 7.5 mM imidazole.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07616

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :C07K 1/16, 1/18, 1/30, 14/81
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/380, 381, 392, 395, 414, 415, 418, 419, 420, 421, 422, 426, 427

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, EMBASE, MEDLINE, INPADOC
search terms: proteinase inhibit?, precipitat? zinc, zncl?, anion exchang? chromatog?

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>----<br>Y | US, A, 4,629,567 (BOLLEN ET AL) 16 DECEMBER 1986, see column 1, lines 1-3; column 2, lines 44-48; column 4, lines 3-39; and column 5, lines 42-60. | 1-2,4,8,17<br>----------<br>3,5-7,9-16,18-20 |
| Y | Biotechnology Letters, Volume 13, Number 4, issued 1991, P. K. Ng et al, "Plasma Protein Recovery From Spent Tissue Culture Fluid", pages 261-264, see page 261, lines 7-24 and page 263, Figure 1. | 3, 5-7, 20 |
| Y | E. L. V. Harris et al, "Protein Purification Methods", published 1989 by IRL Press (Oxford), see pages 154-170, especially page 154, lines 13-45; page 156, lines 1-2; page 161, lines 42-45; and page 164, lines 17-31. | 3, 5-7, 20 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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| 03 AUGUST 1995 | 26SEP 1995 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>NANCY J. DEGEN<br>Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07616

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US, A, 4,540,573 (NEURATH ET AL) 10 SEPTEMBER 1985, see the abstract, lines 1-11; column 6, lines 41-57; column 7, lines 9-45 and lines 50-68 through column 8, lines 1-10 and lines 35-48. | 9-16, 18-20 |
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| Y | Biochemistry, Volume 30, issued 1991, R. Bischoff et al, "Purification and Biochemical Characterization of Recombinant Alpha-1-Antitrypsin Variants Expressed in Escherichia coli", pages 3464-3472, see page 3464, abstract, lines 1-3 and page 3465, column 2, lines 1-15. | 20 |
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| A | US, A, 5,276,141 (KOLBE) 04 JANUARY 1994, see entire document. | 1-20 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07616

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

530/380, 381, 392, 395, 414, 415, 418, 419, 420, 421, 422, 426, 427

Original Paper

Vox Sanguinis

Vox Sang 1998;74:232–241

Received: October 22, 1996
Revised manuscript received: May 15, 1997
Accepted: September 22, 1997

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# Purification of $\alpha_1$ Proteinase Inhibitor from Human Plasma Fraction IV-1 by Ion Exchange Chromatography

**Abstract**

**Background and Objectives:** α-proteinase inhibitor (PI) protects the lungs from proteolytic damage caused by elastase and can be used to treat congenital emphysema. We describe an improved method of purification of $\alpha_1$ PI from redissolved fraction IV-1 paste. **Materials and Methods:** The process used dimethylaminoethyl anion exchange chromatography, sulfopropyl cation exchange chromatography, virus inactivation by dry heat, and tri-n-butyl-phosphate/cholate treatment, followed by a second strong cation exchange chromatography. Optimizations of loading conditions for ion exchange chromatography at small scale (20–60 ml of suspension) are described. Virus inactivation was adjusted to provide the best yield of $\alpha_1$ PI consistent with effective inactivation. The process has been effectively scaled up. **Results:** The final product was approximately 90% pure by SDS-PAGE, with a 60–70% yield from starting fraction IV-1 paste. The process has been characterized by methods including nonreduced SDS-PAGE, $\alpha_1$ PI inhibition assay, and biuret protein assay. **Conclusion:** The method described is an effective way of preparing large quantities of $\alpha_1$ PI from fractionated plasma.

## Introduction

$\alpha_1$ proteinase inhibitor (PI) belongs to the serine proteinase inhibitor (SERPIN) family of proteins and protects the lungs against proteolytic damage caused by elastase [1]. It is secreted by the liver into the circulation and diffuses into tissue spaces, where it inactivates free elastase by forming stable complexes that are rapidly removed from the circulation [2]. Congenital deficiency in $\alpha_1$ PI which results in emphysema [1–6] may be treated by infusion of 4 g of $\alpha_1$ PI per 70-kg person every 2 weeks throughout life [7]. According to the World Health Organization [8], it is estimated that only 4% of $\alpha_1$ PI deficiency patients have been identified and less than that are treated.

$\alpha_1$ PI has 394 amino acids, one cysteine residue, and three carbohydrate side chains, giving an overall molecular weight of 52 kD [1, 9]. The crystal structure of $\alpha_1$ PI cleaved at the P1-P1′ bond has been solved by Loebermann et al. [10]. Eighty percent of the polypeptide chain is arranged in eight well-defined α-helices (A-H) and three large pleated β-sheets (A-C). A critical feature of the molecule is the A β-sheet. Active $\alpha_1$ PI serves as a pseudosubstrate for elastase. Elastase first attacks the reactive center loop by cleavage between P1-P1′ residues at $Met^{358}$-$Ser^{359}$; the resulting aminoterminal polypeptide segment can fold into the A β-sheet, accompanied by a major structural rearrangement to form the $\alpha_1$ PI-elastase complex [1]. The crystal structure of uncleaved $\alpha_1$ PI was published by Song et al. [11]. The most

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0042–9007/98/0744–0232 $15.00/0

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significant difference between the uncleaved and cleaved $\alpha_1$ PI is in the conformation of the reactive loop. Polymerization of active $\alpha_1$ PI can be induced by heat. Heat-polymerized $\alpha_1$ PI and cleaved $\alpha_1$ PI show the same urea transition curve on transverse-urea-gradient-polyacrylamide gel electrophoresis, i.e. failure to unfold, indicating that the A sheet is stabilized in these forms [12].

Several publications have described the purification of $\alpha_1$ PI by chromatography [13–21]: DEAE ion exchange chromatography has been widely used [13, 14, 16, 17] as one of the purification steps. Schultze and Heimburger [19] reported using carboxymethyl cation resin to purify $\alpha_1$ PI from human plasma. Based upon the reported concentration of 1.3 mg/ml of $\alpha_1$ PI in plasma [20], the purity of $\alpha_1$ PI prepared by the latter methodology would appear to be $\leq 40\%$.

Ballieux et al. [21] purified a complex of $\alpha_1$ PI and proteinase-3 from purulent sputum using cation exchange chromatography as one of the three purification steps. Under the pH 7.0 condition used in this study, most of the sputum proteins bound to the resin, but $\alpha_1$ PI and proteinase-3 passed through without binding.

Hein et al. [17] described a clinically approved method for the production of $\alpha_1$ PI. This method employs Cohn fraction IV-1 paste as the starting material; however, Cohn fraction IV-1 paste is precipitated in the presence of 20% ethanol, at pH 5.1, which is denaturing for $\alpha_1$ PI. For this reason, the paste must be dissolved at pH 9 and heated at 45°C for 90 min in order to recover $\alpha_1$ PI activity. The solution is then precipitated with polyethylene glycol followed by chromatography on DEAE Sepharose. The process also incorporates at 10-hour heat treatment at 60°C as a viral inactivation step. Although the $\alpha_1$ PI produced by this methodology is clinically valuable, the yield from IV-1 paste is about 45% and purity is about 60%.

We report here a much needed new process for producing $\alpha_1$ PI that gives both a higher yield and purity and that includes an additional viral inactivation step to ensure the safety of the product. The combination of both solvent detergent treatment and dry heat treatment is necessary to ensure sufficient inactivation of viruses. Solvent detergent treatment is effective against enveloped viruses such as HIV, but is ineffective against non-enveloped viruses such as HAV. Dry heat treatment is effective against both enveloped and non-enveloped viruses. Together, these two methods and other processing steps will provide $>10$ logs of removal of enveloped viruses and $>6$ logs of removal of non-enveloped viruses as recommended by the Paul Ehrlich Institute guidelines [22]. This new process uses DEAE resin for removal of lipoprotein. A strong cation resin is then used at low pH, low salt concentration, and moderate protein concentration. Following two different viral inactivation steps, additional contaminating proteins and inactive $\alpha_1$ PI are removed by a second cation chromatography step. This new process increases the yield to a range of 60–70% and the purity to around 90% by SDS-PAGE.

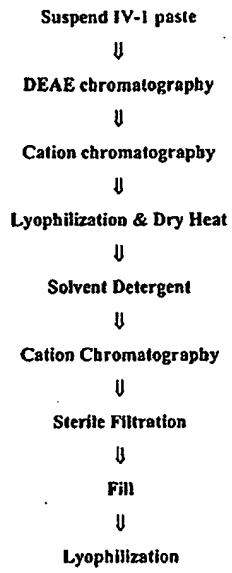


**Fig. 1.** Process flow for $\alpha_1$ PI purification from Cohn fraction IV-1 paste.

## Materials and Methods

*Pilot Scale Purification of* $\alpha_1$ PI

Figure 1 outlines the steps in processing $\alpha_1$ PI from Cohn fraction IV-1 paste. The preparation of Cohn fraction IV-1 paste was as described by Lebing et al. [23]. Frozen human plasma was thawed and the cryoprecipitate removed by centrifugation. Fibrinogen was removed by precipitation in 8% ethanol followed by centrifugation. Immunoglobulins were precipitated by addition of ethanol to 20% and removed by centrifugation as fraction II+III. IV-1 paste was precipitated by adjustment of pH to 5.1 and removed by centrifugation. IV-1 paste was suspended in 0.005 *M* Tris buffer, followed by heating according to Hoffman [24]. The new process comprises one DEAE chromatography, two cation chromatographies, and two viral inactivation steps.

The heat-treated IV-1 suspension was adjusted to conductivity $\leq 5$ mmho/cm with NaCl, and then the pH was adjusted to 8.0. The column, described below, was equilibrated with 20 m*M* dibasic sodium phosphate and 20 m*M* sodium chloride at pH 8.0. Five column vol-

umes of IV-1 suspension were applied to each column volume of DEAE Sepharose fast flow resin (Pharmacia). The suspension was loaded at a linear flow rate of 150 cm/h to a 18 cm diameter × 20 cm high column (Pharmacia), followed by a 3 column volume (CV) wash with equilibration buffer. $\alpha_1$ PI was eluted with 20 m*M* dibasic sodium phosphate and 95 m*M* sodium chloride at pH 8.0. The remaining bound proteins were eluted from the resin with 100 m*M* dibasic sodium phosphate and 1 *M* sodium chloride at pH 8.0. The column was cleaned with 0.5 *M* NaOH and then regenerated with 100 m*M* dibasic sodium phosphate and 1 *M* sodium chloride, followed by 20 m*M* dibasic sodium phosphate and 20 m*M* sodium chloride. The resin may be stored in 0.02 *M* NaOH.

The DEAE eluate was diafiltered against 8 volumes of 20 m*M* monobasic sodium phosphate and 5 m*M* sodium chloride at pH 6.5 at 5°C. This was performed on an SP20 ultrafiltration unit with 10,000 MW cutoff spiral media (Amicon). The diafiltered DEAE eluate was adjusted to a pH of 5.45–5.50 and an absorbance at 280 nm ($A_{280}$) of 3.5. The column, 20 cm diameter × 30 cm high, packed with Macro-Prep High S (BioRad) cation resin, was equilibrated with 20 m*M* monobasic sodium phosphate and 5 m*M* sodium chloride at pH 5.5 at ambient temperature. Three fifths of the DEAE eluate, obtained from DEAE chromatography, were loaded at a linear flow rate of 150 cm/h. $\alpha_1$ PI passes through the column without binding. The flow-through, containing $\alpha_1$ PI, was concentrated to an $A_{280}$ of 40 and adjusted to pH 7.0 with 1 *N* NaOH, and 0.15 *M* sodium chloride was added. The bound proteins were eluted from the cation column with 100 m*M* monobasic sodium phosphate and 1 *M* sodium chloride at pH 5.5. The column was cleaned with 2 CV of 0.5 *M* NaOH and then regenerated with 20 m*M* monobasic sodium phosphate and 5 m*M* sodium chloride at pH 5.5.

At this time, the $\alpha_1$ PI was frozen at –90°C. For the first viral inactivation step, the $\alpha_1$ PI solution was thawed, mixed, adjusted to pH 6.5, histidine was added to give a final concentration of 60 m*M*, and then the solution was lyophilized in trays. The freeze-dry cycle was as follows: hold at atmospheric pressure at a shelf temperature of –25°C for 2 h; decrease shelf temperature to –40°C for another 2 h; apply vacuum to 220 microns and then hold for the rest of the process; heat shelves at –10°C under vacuum for 35.5 h, at which time the shelves were heated to +35°C under vacuum for a further 24 h. The lyophilized material was then heated at 80°C for 72 h to inactivate viruses. Dry heat treatment could lead to limited denaturation of $\alpha_1$ PI; however, the inclusion of a second cation resin removed the denatured $\alpha_1$ PI. Therefore, intermediate dry heat treatment was evaluated instead of dry heat treatment in the final container. It was then dissolved in purified water, and sucrose was added as a stabilizer to a final concentration of 37%. Tri-n-butyl phosphate (TNBP), final concentration 0.3% (w/w), and sodium cholate (0.2%, w/w) were added from a stock solution of 5% (w/w) TNBP and 4% (w/w) cholate. This solvent/detergent treatment inactivates enveloped viruses. After a 3-hour incubation at 30°C, TNBP and cholate were removed by filtration and diafiltration.

The virally inactivated solution was diafiltered against 20 m*M* monobasic sodium phosphate and 5 m*M* sodium chloride at pH 6.5. The diafiltered solution was adjusted to pH 5.5 with 1 *M* acetic acid and loaded onto a second column containing the same resin as the first cation column, 20 cm diameter × 30 cm high, to remove any remaining contaminants, $\alpha_1$ PI denatured by the viral inactivation steps, and TNBP residues. The native form of $\alpha_1$ PI passed through the column with the loading buffer. The denatured $\alpha_1$ PI bound to the column. The collected flow-through was adjusted to pH 7.0, 0.1 *M* sodium chloride. The $\alpha_1$ PI was concentrated by ultrafiltration to 34 mg/ml using a DC-10 ultrafilter with spiral media (Amicon). The solution was filled into the bottles and lyophilized. The following freeze-dry cycle was employed: hold at –25°C for 2 h at atmospheric pressure; decrease temperature to –40°C for another 2 h; apply vacuum to 220 microns for the remainder of the process; heat shelves to +10°C under vacuum for 30 h before the shelves are maintained at +35°C under vacuum for a further 24 h.

*Analytical Assays*

$\alpha_1$ PI activity was determined by inhibition of porcine pancreatic elastase using chromogenic substrate [succinyl-(alanine)$_3$-*p*-nitroanillide] (Sigma) at an absorbance at 405 nm [2]. The assay was performed using a Denly Wellprep 2000 workstation. The samples were prepared by Hamilton Dilutor-Micro Lab 500. The microtiter plates were read with a Molecular Devices Thermomax microtiter plate reader with a 405-nm filter and SoftMax for Windows software version 1.0. Potency was expressed as milligrams of $\alpha_1$ PI per milliliter of sample. A human reference standard plasma (Helena Laboratories) was used. Immunoreactive $\alpha_1$ PI and other proteins were quantified using a laser nephelometer immunoprecipitation assay (Behring Diagnostics) according to the manufacturer's recommendations.

Biuret protein assay was performed at 546 nm [25], using a Denly Wellprep 2000 workstation. The protein concentration was expressed as milligrams of protein per milliliter of sample. A reference standard albumin was used. The specific activity of $\alpha_1$ PI was calculated on the basis of $\alpha_1$ PI activity assay and biuret protein assay.

Polyacrylamide gel electrophoresis was performed using a mini gel system (Novex) with precast 10% polyacrylamide gels. All gels were fixed in 10% TCA and stained with a colloidal blue stain (Integrated Separation Systems). Densitometry was performed using an Ultrascan XL laser densitometer (Pharmacia Diagnostics).

Western blot was performed as follows: proteins were electrophoresed under nonreduced conditions and transferred electrophoretically to a nitrocellulose membrane for 2 h at a constant current of 200 mA. After electrophoretic transfer, the nitrocellulose membrane was blocked overnight with casein in PBS blocker (Pierce) and incubated with an anti-human $\alpha_1$ PI (Pierce) for 1 h at 37°C and 3 h at 4°C. The $\alpha_1$ PI was detected by a chemiluminescent method.

An assay for TNBP was developed by Wayne Zunic and David McAleese [pers. commun.]. Samples containing $<1$ µg/ml TNBP were loaded onto a $C_{18}$ solid phase extraction column. The hydrophobic TNBP partitions into the stationary phase while the hydrophilic protein is washed through the column with water. TNBP and TNPP, which was included as an internal standard, were eluted from the column with hexane and concentrated by evaporation. The TNBP concentration was then determined by gas chromatography using a nonpolar column and flame ionization detection.

An assay for cholate was also developed by Wayne Zunic [pers. commun.]. Cholate in the sample was converted to cholic acid by dilution with hydrochloric acid. This diluted solution was passed through a trifunctionalized $C_{18}$ solid phase extraction column. Cholic acid was retained on the solid phase extraction while the protein in the sample was washed through with hydrochloric acid. The cholic acid was eluted from the solid phase extraction with acetone and collected in a volumetric flask. Acetone was evaporated from the solution with a nitrogen stream evaporator, and the remaining solution is reconstituted with sodium hydroxide. Cholic acid is deprotonated at this high pH, and the cholate ion was separated on an anion exchange column. Detection was accomplished by measuring the current from the oxidation of cholate with a pulsed electrochemical detector.

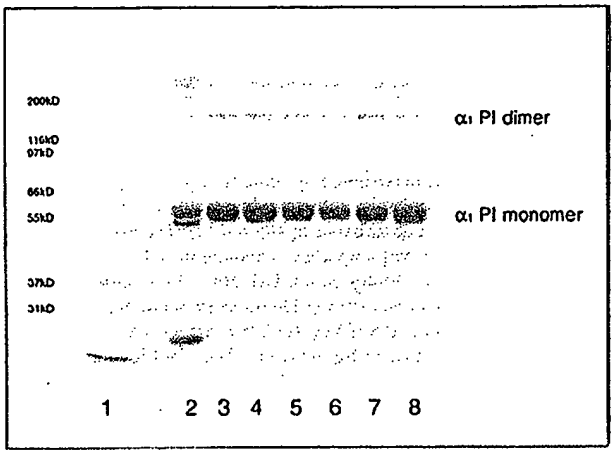


**Fig. 2.** Non-reduced SDS-PAGE analyses of $\alpha_1$ PI in the flow-through of the first cation column. Samples were: molecular weight markers (lane 1), DEAE eluate (lane 2), cation 1 flow-through pool from 1-CV DEAE eluate loading (lane 3), cation 1 flow-through pool from 1.5-CV DEAE eluate loading (lane 4), cation 1 flow-through pool from 2-CV DEAE eluate loading (lane 5), cation 1 flow-through pool from 3-CV DEAE eluate loading (lane 6), cation 1 flow-through pool from 4-CV DEAE eluate loading (lane 7), cation 1 flow-through pool from 5-CV DEAE eluate loading (lane 8). About 15 µg of protein were added to each lane.

## Results

*Optimization of DEAE Anion Chromatography at the Bench Scale*

Table 1 shows the effect of loading different amounts of resuspended IV-1 paste onto the DEAE column on the purity and yield of $\alpha_1$ PI eluted from this column. The loading and wash conditions were selected so that $\alpha_1$ PI binds to the column while contaminants, particularly lipoprotein, flow through the column. There was no detectable $\alpha_1$ PI activity in the flow-through and wash with 4-CV loading. There was 4% yield loss for 5-CV loading. To maximize recovery, 4- to 5-CV loadings were used in the subsequent experiments. As anticipated by displacement chromatography theory [26], the specific activity of $\alpha_1$ PI in the DEAE eluate appeared to increase with larger column loads of resuspended IV-1 paste.

*Optimization of First Cation Chromatography at the Bench Scale*

pH plays a key role in this cation chromatography step. Initial experiments have shown that the pH must be less than 6.0, with an optimum of 5.5. Yield, purity, and capacity are the major considerations for the first cation column. Figure 2 shows the effect of increasing volumes of DEAE eluate on

**Table 1.** Effect of different volumes of resuspended IV-1 paste loading on the purity and yield from DEAE chromatography

| Loading CV | Yield % | Loss in loading % | Specific activity mg $\alpha_1$ PI/mg protein |
|---|---|---|---|
| 4 | 92 | 0 | 0.17 |
| 5 | 86 | 4 | 0.19 |
| 6 | 74 | 18 | 0.25 |

One CV is 16 ml in this experiment. Yield was calculated based on $\alpha_1$ PI activity in the DEAE elution. Yield loss was calculated based on $\alpha_1$ PI activity in the DEAE flow-through and wash. The initial specific activity of IV-1 suspension is about 0.1 mg $\alpha_1$ PI/mg protein.

the purity of $\alpha_1$ PI in the flow-through. The experiment was performed as described in the Materials and Methods section. The starting protein concentration was 4.0 mg/ml. The SDS-PAGE profiles of samples are from pooled flow-through material under different loading conditions. DEAE eluate is given in lane 2. Its profile was comparable to the starting material IV-1 suspension (fig. 6, lane 2). One-CV loading (lane 3) results in 89% $\alpha_1$ PI. The upper band represents $\alpha_1$ PI dimer (see identification of $\alpha_1$ PI monomer and dimer). One-and-a-half- loading (lane 4), 2- loading (lane 5) and 3-CV loading (lane 6) have similar purity in the range of 66–77%. The $\alpha_1$ PI monomer drops to 46% for the 4-CV loading. Based on these results, 3-CV loading was chosen.

*Evaluation of Stabilizers for 80°C Dry Heat Viral Inactivation*

Dry heat at 80°C can inactivate both enveloped and non-enveloped viruses [27]. Dry heat treatment of purified $\alpha_1$ PI with histidine present as stabilizer was also found to inactivate non-enveloped Reovirus type 3 and bovine Parvovirus as anticipated [B. Masecar, pers. commun.]. The latter experiments were performed in vials rather than the slab format described here. It is important to note that the moisture content of the cake may influence the effectiveness of dry heat treatment [B. Masecar and M. Savage, pers. commun.]. A moisture level between 0.3 and 2% was found to provide good viral inactivation ($\leq 6$ $\log_{10}$ reduction of Reovirus, 3.7 $\log_{10}$ reduction for bovine Parvovirus) while preserving 94% of $\alpha_1$ PI activity. Table 2 shows the effect of different stabilizers on activity. There is significant polymer formation using 0.02 *M* sodium phosphate (fig. 3). Both activity recovery and native gel profiles have indicated that histidine (30 and 60 m*M*) stabilized $\alpha_1$ PI better than sodium phosphate (0.02 *M*), glycine (0.1 *M*), or alanine (0.1 *M*). The moisture level was in the range of 0.8–1.0%. Subsequent experiments were performed using 60 m*M* histidine.

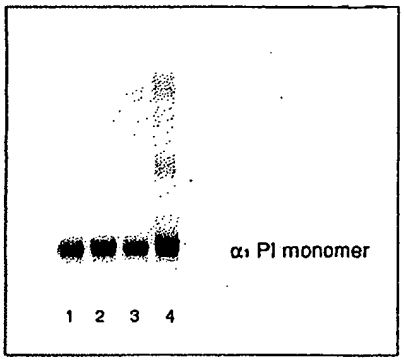


**Fig. 3.** Native gel analyses of dry heat treatment in the presence of different excipients. Samples were: cation 2 flow-through before dry heat in the presence of 60 m*M* histidine (lane 1), cation 2 flow-through after 72 h dry heat treatment in the presence of 60 m*M* histidine (lane 2), cation 2 flow-through before dry heat in the presence of 0.02 *M* sodium phosphate (lane 3), cation 2 flow-through after 72 h dry heat treatment in the presence of 0.02 *M* sodium phosphate (lane 4). About 20 μg of protein were loaded in each lane.

**Table 2.** Effect of different excipients on $\alpha_1$ PI recovery following dry heat treatment

| Excipient | $\alpha_1$ PI recovery after 72 h at 80°C, % |
|---|---|
| 0.02 *M* Na phosphate | 72 |
| 60 m*M* histidine | 88 |
| 30 m*M* histidine | 88 |
| 0.1 *M* glycine | 71 |
| 0.1 *M* alanine | 87 |

The cation 2 flow-through (25 mg/ml) was lyophilized with two conditions for each excipient. One was stored at +5°C while the other set was placed in a 80°C oven for 72 h. Both sets of samples were analyzed for $\alpha_1$ PI activity. The percent recovery of $\alpha_1$ PI was calculated as the ratio of activity remaining in the samples heated at 80°C relative to that observed in the samples stored at 5°C.

**Table 3.** Effect of sucrose/citrate and incubation time on $\alpha_1$ PI recovery following TNBP/cholate treatment

| Method | Test conditions | $\alpha_1$ PI activity recovery, % | | | | |
|---|---|---|---|---|---|---|
| | | 0 h | 0.5 h | 1 h | 2 h | 5 h |
| 1 | no additives | 100 | 103 | 104 | 103 | 97 |
| 2 | sucrose/citrate | 100 | 94 | 97 | 94 | 91 |
| 3 | TNBP/cholate | 100 | 90 | 90 | 74 | 52 |
| 4 | sucrose/citrate, and TNBP/cholate | 100 | 94 | 94 | 91 | 85 |

Test conditions were as follows. In method 1, 5 ml of $\alpha_1$ PI solution were incubated at 30°C, samples were taken at the indicated times. In method 2, the starting sample was first diluted with 2.6 g of sucrose citrate stock solution (0.43 *M* sodium citrate, 7.8 m*M* citric acid, 42.7% sucrose) per gram $\alpha_1$ PI and treated as in method 1. In method 3, samples were diluted with a 7.5% TNBP/7.5% sodium cholate stock solution to give a final concentration of 0.3% TNBP/0.3% sodium cholate. Samples were diluted 1:1,000 prior to the $\alpha_1$ PI activity assay, which minimizes any further damage to the protein by TNBP/cholate. In method 4, the starting sample was first diluted with 2.6 g of sucrose citrate stock solution per gram of $\alpha_1$ PI and then diluted with a 7.5% TNBP/7.5% sodium cholate stock solution to give a final concentration of 0.3% TNBP/0.3% sodium cholate.

*Optimization of TNBP/Cholate Solvent-Detergent Treatment*

Table 3 (lines 3 and 4) shows the effect of incubating $\alpha_1$ PI for different times in the presence of 0.3% TNBP/0.2% sodium cholate. TNBP/cholate alone caused a large initial loss (35%) of $\alpha_1$ PI activity that worsened with time (line 3); there was a further 52% activity loss during the first 5 h. However, the addition of sucrose plus citrate protected the $\alpha_1$ PI against inactivation by TNBP/cholate and reduced the activity loss to 15% over this same 5-hour period (line 4). In the absence of TNBP/cholate, $\alpha_1$ PI was very stable (lines 1 and 2) and exhibited no loss in activity within the assay variation (±10%). Full inactivation of the enveloped virus BVDV was found in both the presence and absence of sucrose [B. Masecar, pers. commun.]. Increasing protein concentration also resulted in higher $\alpha_1$ PI stability (results not shown).

*Conditions of the Second Cation Chromatography*

The method for performing both cation chromatography steps is similar. The first cation chromatography focuses on maximization of yield, while the second cation chromatography emphasizes purity and removes denatured $\alpha_1$ PI. Chromatography was performed at pH 5.5, conductivity ≤2.5 mmho/cm, and protein concentration at $A_{280}$ 4.6. The amount of protein load was $A_{280}$ 7.3/l solution/l resin.

*Identification of $\alpha_1$ PI Monomer and Dimer*

The identity of the upper (MW 158,000) and lower (MW 57,000) SDS-PAGE bands were confirmed as being $\alpha_1$ PI dimer and monomer, respectively, by the following studies. Figure 4A is non-reducing SDS-PAGE. Figure 4B is a West-

ern blot probed with anti-human $\alpha_1$ PI IgG and developed as described in the Materials and Methods. Figure 4C is the control for the Western blot analysis. Both upper and lower bands reacted with the $\alpha_1$-PI-specific antibody, suggesting that both were $\alpha_1$ PI. Reduction of the dimer resulted in a single band on SDS-PAGE with the mobility of $\alpha_1$ PI monomer, consistent with this being the dimer of $\alpha_1$ PI. The Western blot analysis shows that the $\alpha_1$ PI dimer was present in all intermediates throughout the whole process, including the starting material IV-1 suspension. For the IV-1 suspension (fig. 4, lane 1), there was another band above the dimer. The same band was also visible on the control gel (fig. 4C) in the absence of anti-human $\alpha_1$ PI antibody indicating that this bands was unlikely to be $\alpha_1$ PI; it may represent cross-reactivity to human IgG present in the starting material.

*Pilot Scale Process*

Three chromatography steps are involved in the purification of $\alpha_1$ PI from resuspended IV-1 paste (fig. 1). The 280 nm absorbance profile for purification of $\alpha_1$ PI on DEAE is shown in figure 5A. The first broad peak of this profile was the flow-through proteins, which consisted mainly of lipoproteins. The peak at 85 min consisted mostly of transferrin. The third broad peak starting at 94 min contained 25% pure $\alpha_1$ PI with a yield of 88%. The last peak was eluted during cleaning of the column and contained 5% of the applied $\alpha_1$ PI.

The absorbance profile for the purification of $\alpha_1$ PI on the first cation column is shown in figure 5B. The peak from 52 to 102 min contained 73% pure $\alpha_1$ PI with a yield of 96%. The increase in UV absorbance during the loading was accompanied by an increase in pH from 5.5 to 5.9.

The absorbance profile for the purification of $\alpha_1$ PI on the second cation column is shown in figure 5C. The peak from 58 to 93 min contains 92% pure $\alpha_1$ PI with a yield of 99%. The peaks eluted during the cleaning phase in figures 5B and 5C represented proteins bound more tightly to the resin, including denatured $\alpha_1$ PI. Residual TNBP present in the $\alpha_1$ PI preparation was found to bind to the matrix of the resin. This residual TNBP was subsequently removed from the resin by cleaning with 0.5-CV of 90% ethanol. This step was started at 116 min.

A summary of the overall recoveries and purities in the key steps of the process is given table 4. Yields from the three chromatography steps were 88% (DEAE), 96% (1st cation) and 99% (2nd cation). The overall recovery was 63%. The biggest individual loss occurred at the dry heat stage. Figure 6 shows an SDS-PAGE gel of the product at different stages in processing. There were readily apparent purity increases after cation 1 (lane 4) and cation 2 (lane 5).

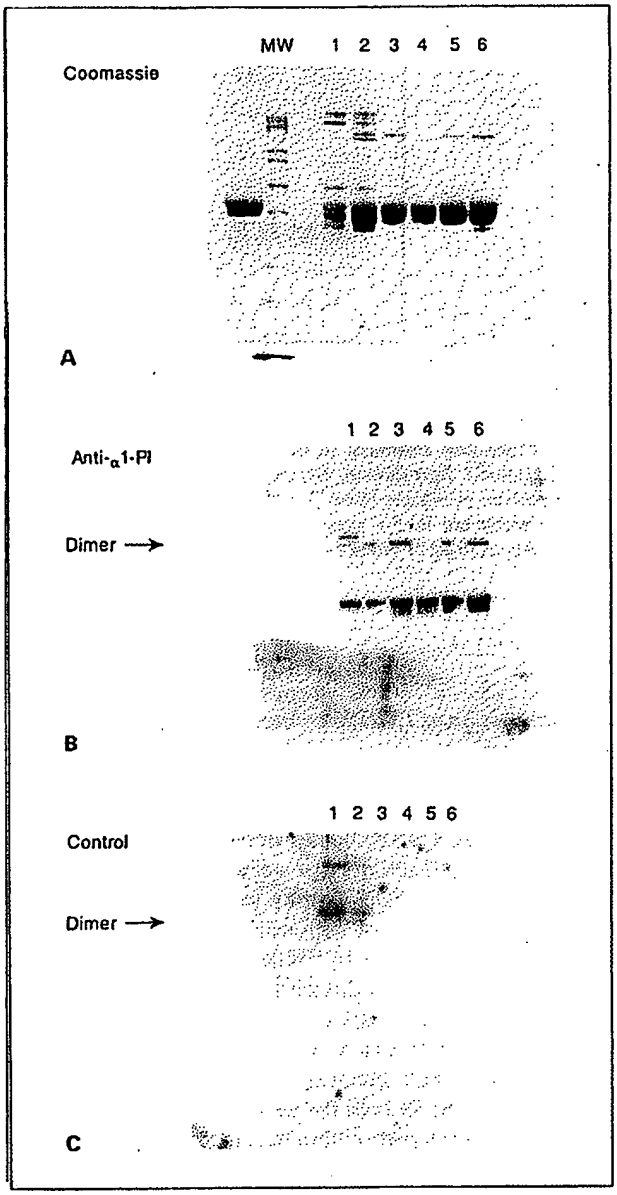


**Fig. 4.** Western blot analyses of $\alpha_1$ PI monometer and dimer for intermediate steps during processing. **A** Non-reduced SDS-PAGE. **B** Western blot probed with anti-human $\alpha_1$ PI IgG from mouse and goat anti-mouse IgG conjugated with alkaline phosphatase. **C** Control for the Western blot probed with secondary antibody goat anti-mouse IgG conjugated with alkaline phosphatase. The molecular weight markers are given in lane MW. Samples were: IV-1 suspension (lane 1), DEAE eluate (lane 2), cation 1 flow-through (lane 3), postpasteurized cation 1 flow-through (lane 4), cation 2 flow-through (lane 5), current product Prolastin (lane 6).

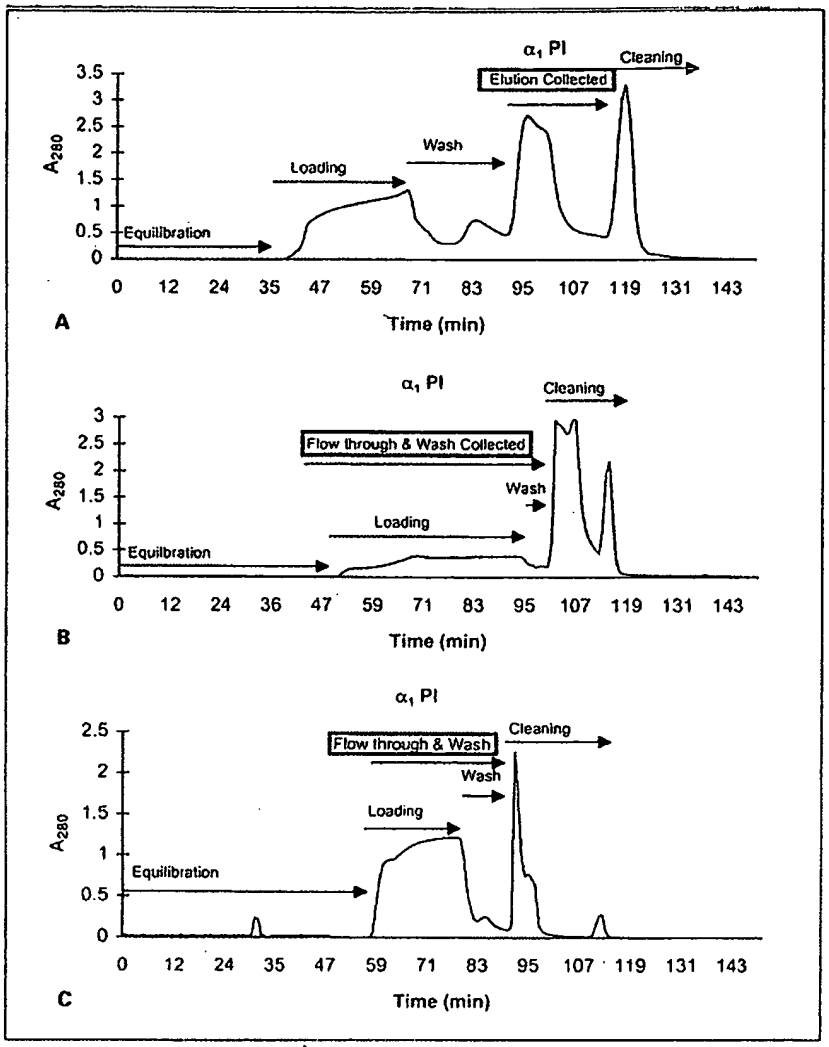


**Fig. 5.** DEAE chromatography, cation 1 chromatography and cation 2 chromatography of $\alpha_1$ PI at the 10-liter scale. Absorbance profile (280 nm) for purification of $\alpha_1$ PI on DEAE column (**A**), on first cation column (**B**), and on second cation column (**C**). Equilibration, loading, washing, elution and cleaning portions of each are shown by arrows and were performed as described. $\alpha_1$ PI indicates the elution position of this protein as determined by $\alpha_1$ PI activity assay.

**Table 4.** Recovery and purity of $\alpha_1$ PI from a pilot scale process

| Step | Total $\alpha_1$ PI g | Yield from IV-1 suspension % | Yield from previous step % | $\alpha_1$ PI monomer by SDS-PAGE | Specific activity mg $\alpha_1$/mg protein |
|---|---|---|---|---|---|
| IV-1 suspension | 282 | 100 | N/A | 14 | 0.11 |
| DEAE eluate | 247 | 88 | 88 | 19 | 0.25 |
| UF cation 1 | 238 | 84 | 96 | 72 | 0.73 |
| Dry heat | 196 | 70 | 82 | | 0.71 |
| TNBP/cholate | 194 | 69 | 99 | | 0.7 |
| Cation 2 | 197 | 70 | 102 | 90 | 0.92 |
| Lyophilization | 179 | 63 | 91 | 89 | 0.83 |

Results are given from one representative run at pilot scale. Recoveries of individual steps are consistent with experiments performed at the bench scale. The total volume of IV-1 suspension was 235 liters. UF = Ultrafiltration.

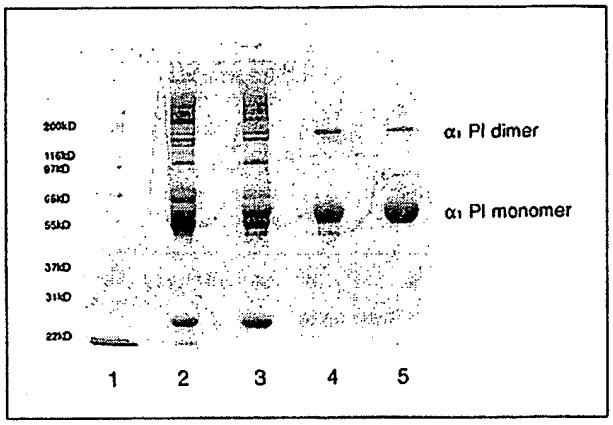


**Fig. 6.** Non-reduced SDS-PAGE analyses of 10-liter scale intermediates. Samples were: molecular weight markers (lane 1), IV-1 suspension (lane 2), DEAE eluate (lane 3), cation 1 flow-through (lane 4), and cation 2 flow-through (lane 5). About 20 μg of protein were loaded in each lane.

**Table 5.** Purity of cation 2 flow-through material as measured by immunonephelometry

| Protein | Concentration mg/ml |
|---|---|
| $\alpha_1$ PI | 25.5 |
| IgA | 0.87 |
| $\alpha_1$ acid glycoprotein | 0.11 |
| Transferrin | <0.09 |
| Albumin | <0.02 |
| IgG | <0.004 |
| Haptoglobin | <0.07 |
| IgM | <0.04 |
| Ceruloplasmin | <0.02 |
| $A_1$ apolipoprotein | <0.05 |
| Fibrinogen | <0.15 |
| Fibronectin | <0.02 |
| ATIII | <0.03 |

Table 5 lists the protein composition of the final product determined by immuno-nephelometry. The major contaminants were IgA and $\alpha_1$ acid glycoprotein, representing 3.4 and 0.4% of the content of $\alpha_1$ PI.

## Discussion

We have described a new process for $\alpha_1$ PI production which includes two viral inactivation steps. This process provides a final product purity of at least 87% $\alpha_1$ PI monomer and 3% $\alpha_1$ PI dimer, as determined by SDS-PAGE analysis. The specific activity of $\alpha_1$ PI obtained from this process was 0.83 g $\alpha_1$ PI/g protein. The overall yield from resuspended IV-1 paste to freeze-dried final product was 63%. Overall, both purity and yield have exceeded previously described processes [17, 28]. This process also includes two established methods of viral inactivation: dry heat and solvent-detergent treatment. Together, these two inactivation methods plus additional clearance throughout the process assure compliance with all current guidelines for the viral inactivation of plasma-derived products.

$\alpha_1$ PI has only one cysteine residue, $Cys^{232}$, which is located in a cavity on the surface of the molecule and is available for SH-SS interchange reactions both in vivo and in vitro [29]. Conformational changes induced by modification of $Cys^{232}$ directly affect the active site loop and alter the affinity of $\alpha_1$ PI for elastase [29, 30]. Our experiments have indicated that the specific activity of $\alpha_1$ PI correlates with the percentage of monomer detected by size exclusion HPLC. Based on these observations, the dimer probably has no activity in our assay method; therefore, the actual activity of $\alpha_1$ PI in our preparation is likely to be underestimated. The formation of the dimer is reversible (data not shown) and has no known clinical significance.

$\alpha_1$ PI is an electrophoretically heterogeneous protein with eight different bands in isoelectric focusing. More than 75 different alleles have been identified [31]. In the fractionation industry, plasma pools are composed of units from many different donors; thus, many different isomers of $\alpha_1$ PI are likely to be represented in our starting material. Based on the lack of $\alpha_1$ PI activity binding to the cation columns, this novel purification technique appears to efficiently separate at least all the major $\alpha_1$ PI variants from other proteins, but may not distinguish between different variants.

Ion exchange chromatography was chosen because of its high binding capacity and relatively low cost. DEAE resin is frequently used for $\alpha_1$ PI purification, but the purity of $\alpha_1$ PI eluted from DEAE resin with IV-1 suspension as starting material is low. The DEAE column removes more than 50% of the lipoprotein in the flow-through. It also removes $\alpha_1$ acid glycoprotein. The removal of these impurities substantially increases the capacity of the cation column.

The selectivity of the cation resin (pH 5.5) contributes to the high purity. Low pH plays a key role in the success of this chromatographic step. $\alpha_1$ PI has an isoelectric point which ranges from pH 4.4 to 4.7 according to different phe-

notypes [31]. Therefore, $\alpha_1$ PI carries a net negative charge at pH 5.5 and flows through the column. One of the identified contaminants, $\alpha_1$ acid glycoprotein, can also flow through the column because of its low pI, 2.7 [32]. The major contaminant, IgA, is partially co-eluted with $\alpha_1$ PI on the DEAE column and is separated on the cation column. The small amount of IgA flowing through the cation column probably represents breakthrough of IgA. Higher removal of IgA is possible by decreasing the loading of this column. However, this affects the yield and capacity of the process.

For safety considerations, at least two different viral inactivation steps need to be incorporated into the production of plasma-derived products. This assures compliance with the PEI guidelines for viral clearance of plasma products. These guidelines state that the clearance of enveloped virus must be at least 10 logs including two individual steps each of at least 4 logs. Non-enveloped viral clearance must be at least 6 logs and should include a single step of at least 4 logs. Both dry heat treatment at 80°C for 72 h and TNBP/cholate treatment can result in some degree of $\alpha_1$ PI denaturation due to polymerization or to structural changes. Two strategies for dry heat treatment have been evaluated. The first uses a process intermediate while the second heats the final container. Both have their strengths and weaknesses. The intermediate dry heat treatment is more expensive and difficult to perform from a production perspective though removal of any heat-denatured material is possible during subsequent processing, e.g. the second cation column removes heat denatured $\alpha_1$ PI. Terminal dry heat is easier to perform from a manufacturing perspective, but offers no opportunity to remove denatured protein from the final container. It is possible that some isoforms of $\alpha_1$ PI exhibit different stability. Loop-sheet polymerization can cause loss of activity. Schulze et al. [33] demonstrated that native antitrypsin heated at 60°C for 2 h forms polymers. Polymerization also occurs when $\alpha_1$ PI is heated at 48°C for 15 h [12]. The polymers are noncovalently assembled. Therefore, monomers can be regenerated from the polymers by treatment with 2% SDS in the absence of thiol-reducing agents [34]. The probable mechanism of heat-induced polymerization is due to interactions between components of the A-sheet and reactive site loop of adjacent $\alpha_1$ PI molecules [12]. This mechanism may be able to explain the limited denaturation observed during dry heat treatment at 80°C. It is also possible that polymers may be formed during solvent-detergent treatment. Formation of polymers can also be analyzed by native PAGE.

The second cation chromatography step is incorporated for removal of the denatured $\alpha_1$ PI. Heat and solvent-detergent treatments can cause denaturation of $\alpha_1$ PI [30]. This may be due to a conformational change where negative charge groups on the molecule form a salt bridge within the molecule or with adjacent $\alpha_1$ PI molecules, resulting in an increased affinity for the cation resin. More than 95% of the TNBP and cholate have been removed by filtration and diafiltration prior to the second cation chromatography column. This chromatography step also functions to remove residual TNBP.

Although $\alpha_1$ PI has been purified from recombinant sources, plasma remains the only source for producing licensed product. In the plasma industry, Cohn fractionation is still the backbone for purification of a variety of products, such as IgG, albumin, fibrinogen, and ATIII. The starting concentration of $\alpha_1$ PI in the plasma is about 1.3 mg/ml [20]. The recovery of $\alpha_1$ PI in Cohn fraction effluent I is around 95% of $\alpha_1$ PI in the plasma. The recovery of $\alpha_1$ PI at Cohn fraction effluent II+III stage is around 90%, while the recovery of $\alpha_1$ PI at the IV-1 paste stage significantly drops to around 25% of $\alpha_1$ PI in the plasma. The recovery of $\alpha_1$ PI can be increased to about 50% in resuspended IV-1 paste through incubation at pH 9.4 and 40°C for 1 h, which is the starting material of this study. The recovery of 50% still represents a significant loss. It is clear that affinity capture of $\alpha_1$ PI from either effluent I or effluent II+III is more desirable than starting with the resuspended IV-1 paste. We anticipate that the process presented here will be useful in processing $\alpha_1$ PI captured by the affinity capture step. It is of interest to note that $\alpha_1$ PI can be purified from effluent II+III by cation chromatography. In addition, the present process of purification and viral inactivation may play a role in processing $\alpha_1$ PI from future sources, including recombinant and transgenic systems.


## Acknowledgements

The authors would like to thank the following Bayer-Clayton staff: Woody Wood, Pam Amos, Susan Trukawinski, Anthony Klos, Barbara Masecar, George Baumbach, Doug Sakowski and John Buck for assistance with pilot scale separations; George Baumbach for providing Western blot; Barbara Masecar and Margaret Savage for viral validation; support from Bayer, Clayton QA and Production departments. We also thank Tom Zimmerman for the editorial comments.

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AIR2003

The meeting has been supported by generous grants from

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PROGRAMME

# INTERNATIONAL MEETING ON ALPHA1-ANTITRYPSIN DEFICIENCY

WORLD
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SPAIN
11-12-13 JUNE
2003

ALPHA ONE INTERNATIONAL REGISTRY A. I. R.

ALPHA ONE INTERNATIONAL REGISTRY A. I. R.

# Alpha One International Registry (AIR)

The Alpha One International Registry (AIR) is a multinational research project, representing a combined research effort of nearly 20 countries. It was formed in response to a recommendation of the World Health Organization at a meeting on alpha1-antitrypsin (AAT) deficiency in March 1996.

Representatives from the member countries meet at least twice per year to discuss AAT research and the progress of AIR.

Each member country has a National Registry of patients with AAT deficiency, and contributes information about individuals with AAT deficiency within the country to an international database in Mälmo, Sweden. The information sent to Sweden is identified only by a number, and no information is sent that can be traced to individual people.

This international cooperative effort can be expected to stimulate medical research through resources that are only available through multinational cooperation. The AIR members are actively studying ways to improve detection of the disease, ways to follow the course of the lung disease, and ways to improve treatment of the disease.

## Chairman of the Meeting

Robert A. Stockley (UK, chairman of A.I.R.)

## Scientific Coordinators

Marc Miravitlles (Spain)
Niels Seersholm (Denmark)

## Scientific Committee

Robert A. Stockley (UK)
Marc Miravitlles (Spain)
Maurizio Luisetti (Italy)
Bruce C. Trapnell (USA)
Niels Seersholm (Denmark)
David A. Lomas (UK)
Dino Hadzic (UK)

## Invited speakers and chairs

GILL AINSLE (Cape Town. South Africa)
BRUNO BALBI (Pavia. Italy)
JOAN A. BARBERÀ (Barcelona. Spain)
MARK L. BRANTLY (Gainesville. Florida. USA)
EDWARD CAMPBELL (Salt Lake City. Utah. USA)
ROBIN CARRELL (Cambridge. UK)
MORTEN DAHL (Copenhagen. Denmark)
ASGER DIRKSEN (Copenhagen. Denmark)
JUAN DOMÍNGUEZ-BENDALA (Miami. Florida. USA)
SARAH EVERETT (Boston. Massachusetts. USA)
SHANE FITCH (Cádiz. Spain)
DINO HADZIC (London. UK)
JOHN HUMPHRIES (Research Triangle Park. North Carolina. USA)
SABINA JANCIAUSKIENE (Copenhagen. Denmark)
CLAES-GORAN LÖFDHAL (Lund. Sweden)
DAVID A. LOMAS (Cambridge. UK)
MAURIZIO LUISETTI (Pavia. Italy)
MARC MIRAVITLLES (Barcelona. Spain)
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CLAUS VOGELMEIER (Margburg. Germany)
MARK A. ZERN (Sacramento. California. USA)

# WEDNESDAY 11th June

## Alpha-1 International Patient Congress

| | |
|---|---|
| 9:00-9:10 | Conference Introduction<br>*Sarah Everett/ Shane Fitch* |
| 9:10-10:00 | Delegates Introduction: Problems Faced by Alpha-1 Patients and Families in their Country |
| 10:00-11:15 | Panel Discussion: Existing Resources for Alpha-1 Patients and their Families |
| 11:15-11:45 | Morning Break: Exhibits |
| 11:45-12:45 | Scientific Presentation<br>*Chairman: Gordon L. Snider* |
| | Molecular Basis for AAT Deficiency<br>*David A. Lomas* |
| | Lung Disease due to AAT Deficiency<br>*Jan Stolk* |
| | Liver Disease due to AAT Deficiency<br>*Jeffrey H. Teckman* |
| 12:45-13:00 | Patient Group Photo |
| 13:00-14:15 | Lunch |
| 14:15-16:15 | Scientific Presentation Continued<br>*Chairman: Sandy Sandhaus* |
| | Current and Future Therapies<br>The European View<br>*Marc Miravitlles* |
| | View from the Americas<br>*Sandy Sandhaus/Bruce C. Trapnell* |
| | AIR: The Role of the Research Registry<br>*Marc Miravitlles* |
| | The Patient and the Research Registry<br>*Charlie Strange* |
| | The Alpha-1 Sib Pairs Study<br>*Edwin K. Silverman/Robert A. Stockley* |
| 16:15-16:30 | Afternoon Break: Exhibits |
| 16:30-18:00 | Discussion: Future Initiatives of Alpha-1 Patients International |
| 18:00 | Adjournment |
| 20:30 | Cocktail |



# THURSDAY 12th June

## Session I Two years in review

*Chairmen: Gerard M. Turino/Asger Dirksen*

| | |
|---|---|
| 8:30-9:00 | Pathophysiology: exacerbations and inflammation<br>*Robert A. Stockley* |
| 9:00-9:30 | Liver disease in AATD<br>*Jeffrey H. Teckman* |
| 9:30-10:00 | Clinical management<br>*Sandy Sandhaus* |
| 10:00-10:30 | Coffee break/Poster viewing |

## Session II Basic science: proteins and proteases

*Chairmen: David A. Lomas/Mark L. Brantly*

| | |
|---|---|
| 10:30-10:50 | Protein-protein interaction in the regulation of AAT inhibitory capacity<br>*Maurizio Luisetti* |
| 10:50-11:10 | Cytokines in the pathogenesis of COPD. What is really important?<br>*Jean-Michel Sallenave* |
| 11:10-11:30 | Proteases and animal models<br>*Bob Senior* |
| 11:30-11:55 | Discussion |
| 11:55-12:10 | Break |

## Session III Laurell lecture

*Chairman: Robert A. Stockley*

| | |
|---|---|
| 12:10-12:30 | Laurell's syndrome<br>*John Humphries* |
| 12:30-13:00 | Laurell Lecture<br>*Robin Carrell* |
| 13:00-14:15 | Lunch |

## Session IV Basic science update

*Chairmen: Bruce C. Trapnell/Claus Vogelmeier*

| | |
|---|---|
| 14:15-14:35 | Pulmonary arterial dysfunction in COPD<br>*Joan Albert Barberà* |
| 14:35-14:55 | Gene therapy for AAT deficiency<br>*Terry Spencer* |
| 14:55-15:15 | Stem cell potential in AAT deficiency<br>*Juan Domínguez-Bendala* |
| 15:15-15:35 | Liver cell immortalization for the therapy of AAT deficiency<br>*Mark A. Zern* |
| 15:35-15:55 | Patterns of emphysema in alpha-1-antitrypsin deficiency patients and usual COPD<br>*Saher Shaker* |
| 15:55-16:20 | Discussion |
| 16:20 | Adjournment |
| 20:00 | Dinner |

# FRIDAY 13th June

## Session V Liver disease

*Chairmen: Dino Hadzic/Jeffrey H. Teckman*

| | |
|---|---|
| 9:00-9:20 | Liver disease in PiSS/PiSZ<br>*Dino Hadzic* |
| 9:20-9:40 | Processing of Z antitrypsin within the endoplasmic reticulum of hepatocytes<br>*Richard Sifers* |
| 9:40-10:00 | Immunological detection of Z antitrypsin polymers in the liver and circulation<br>*Sabina Janciauskiene* |
| 10:00-10:20 | Discussion |
| 10:20-10:45 | Coffee break/Poster viewing |

## Session VI Other phenotypes

*Chairmen: Edwin K. Silverman/Erich Russi*

| | |
|---|---|
| 10:45-11:05 | High prevalence of rare deficient variants in Italy<br>*Bruno Balbi* |
| 11:05-11:25 | Impact of PiMZ in COPD<br>*Morten Dahl* |
| 11:25-11:45 | Natural history of lung disease in PiSZ individuals<br>*Niels Seersholm* |
| 11:45-12:05 | Presence of null phenotypes in AAT deficiency<br>*Edward Campbell* |
| 12:05-12:30 | Discussion |
| 12:30-13:45 | Lunch |

## Session VII Registries update

*Chairmen: Gil Ainsle/Mark L. Brantly*

| | |
|---|---|
| 13:45-14:05 | U.S. registry update<br>*Charlie Strange* |
| 14:05-14:25 | Update on A.I.R. registry<br>*Claes-Goran Löfdhal* |
| 14:25-14:45 | Swedish birth cohort<br>*Tomas Sveger* |
| 14:45-15:00 | Discussion |





## Session VIII Open Session on clinical issues

*Chairmen: Jan Stolk/Eeva Piitulainen*

15:00-16:00 Open Discussion

- A complete single nucleotide polymorphism (SNP) map of the alpha-1-antitrypsin (AAT) gene.
  *Sally Plummer, Tamar Guetta-Baranés, Kevin Morgan, Noor Kalsheker. Institute of Genetics, University of Nottingham, Nottingham NG7 2 UH, England, UK.*
- Patterns of emphysema in alpha-1-antitrypsin deficiency patients and usual COPD.
  *Saher Shaker, Asger Dirksen, Lars Laursen, Niels Maltbaek, Lars Christensen, Ulla Sander, Niels Seersholm, Axel Kok-Jensen. Dept. of Respir. Med., Gentofte Hospital, Hellerup, Denmark.*
- Assessment of expiratory flow limitation in alpha1-antitrypsin deficiency patients suffering from COPD.
  *Luciano Corda, Enrica Bertella, Enrico Boni, Michele Guerini, Claudio Tantucci.*
  *Prima Divisione di Medicina Generale, Spedali Civili - Chair of Respiratory Disease University of Brescia - Brescia, Italy.*
- Alpha-1 proteinase inhibitor: adjustment of individual therapeutical doses.
  *M. López, N. Muñoz, M. Costa, M. Massot, J.I. Jorquera. Research and Development Area, Instituto Grifols S.A., Barcelona, Spain.*
- Blood Pressure, Risk of Ischemic Cerebrovascular and Ischemic Heart Disease, and Longevity in a1-antitrypsin deficiency. The Copenhagen City Heart Study.
  *Morten Dahl, Anne Tybjærg-Hansen, Henrik Sillesen, Gorm Jensen, Rolf Steffensen, Børge G. Nordestgaard. Herlev, Gentofte, Hilierød, Hvidovre, Bispebjerg and Copenhagen University Hospitals, Copenhagen, Denmark.*

| | |
|---|---|
| 16:00-16:20 | Is replacement therapy efficacious?<br>*James K. Stoller* |
| 16:20-16:30 | Discussion |
| 16:30 | Adjournment |
| 20:00 | Dinner |

# Oral presentations

# Poster presentations

FRIDAY 13th JUNE
15:00-16:00 h

- **A complete single nucleotide polymorphism (SNP) map of the alpha-1-antitrypsin (AAT) gene.**

*Sally Plummer, Tamar Guetta-Baranes, Kevin Morgan, Noor Kalsheker*
*Institute of Genetics, University of Nottingham, Nottingham, England, UK.*

AAT deficiency is the only well recognised genetic risk factor for developing COPD but less than 1% of patients are actually AAT deficient. It has been suggested that the COPD pathogenesis is a result of an imbalance between proteinases and proteinase inhibitors in the lung, and genetic variants of AAT have been associated with COPD. We screened the AAT gene for SNPs by a combination of direct sequencing in 44 individuals and alignment using ClustalW. This information has been used to construct a complete SNP map covering all exonic regions, known regulatory regions and 1000bp of both 5' and 3' flanking sequence. Sixteen polymorphisms have been identified in AAT, ten of which were present in the databases in the public domain. All of these sites are currently being genotyped in control samples and COPD patients to estimate the common haplotypes by maximum likelihood analysis. This will permit identification of both the informative haplotype tag SNPs and the redundant non-informative SNPs, as well as providing important linkage disequilibrium data thereby allowing a reduction of the number of loci that need to be included in future association studies, providing cost and time benefits.

*This work was supported by the Eurolung Consortium QLG1-CT-2001-01012.*

- **Patterns of emphysema in alpha-1-antitrypsin deficiency patients and usual COPD.**

*Saher Shaker, Asger Dirksen, Lars Laursen, Niels Mallback, Lars Christensen, Ulla Sander, Niels Seersholm, Axel Kok-Jensen*
*Dept. of Respir. Med., Gentofte Hospital, Hellerup, Denmark.*

The 1985 report of the National Heart, Lung and Blood Institute Division of Lung Disease Workshop (1) defines emphysema as "a condition of the lung characterized by abnormal, permanent enlargement of airspaces distal to the terminal bronchiole, accompanied by the destruction of their walls, and without obvious fibrosis". In 1990 Flenley suggested that repeated quantitative CT might assess the progression of emphysema more accurately, than by measuring the FEV1 (2). Recently this suggestion has been supported by findings in subjects with alpha-1 antitrypsin deficiency and moderate to severe emphysema. In these subjects CT derived lung density measurements proved to be twice as sensitive as FEV1 for monitoring the progress of emphysema (3). But the most important drawback of CT is radiation exposure, which could limit its use to monitor the progression of emphysema.
In the current study we have evaluated the reproducibility of CT lung density in 50 patients with moderate COPD: 25 with alpha-1 antitrypsin deficiency (AATD) and 25 current smokers with usual COPD. With an interval of 2 weeks, the participants had 3 low-dose multi-slice CT scans using 3 different radiation doses (20%, 10% and 5% of the conventional CT dose of 200 mAs). Images were reconstructed using 3 different algorithms (low, medium and high spatial resolution). The results confirm good reproducibility of CT densitometry (15th percentile (st. dev.): 2-4 HU), which has even been improved by the multi-slice technique that allows a full lung volume scan to be performed within 10 seconds, i.e. during a breath hold. Lung density was significantly lower and reproducibility significantly better among patients with AATD as compared to those with usual COPD with similar pulmonary function. Reproducibility was independent of radiation dose and reconstruction algorithm. We believe that CT lung density measurements in patients with COPD are highly reproducible and can be used in future studies on disease progression and monitoring of the disease modifying treatments.

*References:*
*1. Snider GL et al. The definition of emphysema; Am Rev Respir Dis 1985, 132: 182-85.*
*2. Flenley DC. Diagnosis and follow-up of emphysema; Eur Respir J. 1990. 3 (suppl. 9):5-8.*
*3. Dirksen et al. A randomized clinical trial of a1-antitrypsin augmentation therapy; AJRCCM 1999, 160:1468-72.*

- **Assessment of expiratory flow limitation in alpha1-antitrypsin deficiency patients suffering from COPD.**

*Luciano Corda, Enrica Bertella, Enrico Boni, Michele Guerini, Claudio Tantucci*
*Prima Divisione di Medicina Generale, Spedali Civili - Chair of Respiratory Disease University of Brescia - Brescia, Italy.*

Airway obstruction may lead to expiratory flow limitation (EFL) that, promoting dynamic hyperinflation (DH), is related to chronic dyspnea and exercise tolerance in COPD. Different mechanisms play a role for airflow obstruction in different COPD phenotypes, possibly influencing the development of EFL.
The aim of the study was to verify if EFL at rest was more frequently related to panacinar emphysema in alpha1-antitrypsin-deficiency (AATD) than in centro-acinar emphysema or chronic bronchitis.
Twenty-five COPD patients with similar FEV1 (10 with severe AATD - group A, 8 with prevalent centro-acinar emphysema - group E, and 9 with prevalent chronic bronchitis - group CB), underwent PFT and HRCT. EFL was assessed by NEP method, changes in DH by measuring inspiratory capacity (IC), both in seated and supine position before and after inhaled salbutamol (400$\mu$g).
EFL was found in all groups, but it was more frequently observed in supine position in A (70% vs 30% when seated, $p=0.07$) and E (75% vs 25% when seated, $p<0.05$) as compared to CB (57% vs 43% when seated, n.s.). Salbutamol increased IC, thus decreasing DH, mainly in supine position, in a number of COPD patients, mostly in CB and E, allowing the disappearance of EFL in few of them.



Scientific Programme AIR

- **Alpha-1 proteinase inhibitor: adjustment of individual therapeutical doses.**

*M. López, N. Muñoz, M. Costa, M. Massot, J.I. Jorquera*
*Research and Development Area. Instituto Grifols S.A. Barcelona, Spain.*

This preliminary in vitro study is aimed at adjusting the A1PI doses to the patients' individual requirements, by unifying the content of several vials into a single container, under sterile conditions.
Three lots of A1PI were stored in polypropylene bags for intravenous use (Griflex bags), mimicking the worst case conditions with regard to contact surface area/volume ratio.
Samples were subjected to temperatures of 5 ºC, 30 ºC/60% HR and 40 ºC/60% HR.
Elastase inhibitory activity (chromogenic), protein (Bradford), pH, turbidity, molecular distribution, plastic additives (HPLC) and metals (atomic absorption) were evaluated at different time points.
The results at 5 ºC and 30 ºC do not show variations after 15 days. The activity remains constant and the molecular distribution does not show alterations of the product profile. No migration of metals or plastic additives is detected (even at 40 ºC).
The results obtained at 5 ºC, 30 ºC and 40 ºC support the possibility of preparing specific therapeutical doses into polypropylene (e.g.: Griflex) bags for intravenous use, under sterile conditions.

- **Blood Pressure, Risk of Ischemic Cerebrovascular and Ischemic Heart Disease, and Longevity in a1-antitrypsin deficiency. The Copenhagen City Heart Study.**

*Morten Dahl, Anne Tybjærg-Hansen, Henrik Sillesen, Gorm Jensen, Rolf Steffensen, Børge G. Nordestgaard. Herlev, Gentofte, Hillerød, Hvidovre, Bispebjerg and Copenhagen University Hospitals, Copenhagen, Denmark.*

Background: Because elastase in a1-antitrypsin deficiency may attack elastin in the arterial wall, we tested wether a1-antitrypsin deficiency is associated with reduced blood pressure, risk of ischemic cerebrovascular disease (ICVD) and ischemic heart disease (IHD), and longevity.
Methods and Results: We genotyped 7963 control subjects from the adult general population of Denmark, 1131 Danish patients with ICVD, and 2221 Danish patients with IHD. Compared with MM/MS individuals, systolic blood pressure was lower by 15 mmHg in ZZ homozygotes (n=6, P=0.03) and 9 mmHg in MZ heterozygotes with IHD (n=39, P=0.02). Odds ratios for ICVD and IHD in MZ versus MM/MS individuals were 0.70 (0.51 to 0.96) and 0.77 (0.61 to 0.98). Finally, mean ages of MZ and MM/MS control subjects were 58 and 56 years (Mann-Whitney: P=0.008), and relative a1-antitrypsin MZ genotype frequencies increased from 20 to 93 years among control subjects (c2, P=0.002).
Conclusions: ZZ a1-antitrypsin deficiency and MZ intermediate deficiency in the context of IHD are associated with reduced blood pressure, and MZ is associated with reduced risk of ICVD and IHD. Because MZ heterozygosity was associated with increased age, MZ heterozygosity could be a beneficial condition.

- **Alpha-1 Antitrypsin Deficiency (AATD) and Chronic Obstructive Pulmonary Disease (COPD).**

*Gordon L. Snider*
*Boston University School of Medicine and VA Boston Healthcare System. Boston, Massachussets. USA.*

Diseases are due to interactions between the host and one or more causes of disease, thus making each patient's disease unique. Where affected patients have common features we have developed diagnoses as names for diseases. The defining characteristics may be an etiologic agent, a disorder of structure or function or a consistent syndrome. Diagnostic criteria are features of the disease that are found by empiric research to best distinguish the disease from other diseases that resemble it. Definitions are not critical to doing good clinical or scientific work; diagnostic criteria are critically important for both sound clinical and research work. COPD may be defined (in a variant of the GOLD proposal) as a disease state characterized by incompletely reversible, progressive airflow obstruction that is associated with inflammation in the lungs due to prolonged exposure to tobacco smoke and other noxious particles and gases. Persons with severe AATD have an additional, genetic susceptibility factor above the risk factors of usual COPD. Clinically the disease presentations are more alike than they are different, except for the more rapid progression of emphysema in AATD; panacinar emphysema predominates in AAT-COPD. It thus seems convenient to develop appropriate diagnostic criteria and to speak of usual and AAT-COPD.

- **Alpha-1 proteinase inhibitor (A1PI): in vitro characterisation before and after aerosolisation.**

*M. López, N. Muñoz, M. Massot, J.I. Jorquera*
*Research and Development Area. Instituto Grifols S.A. Barcelona, Spain.*

A1PI concentrates could be administered as an aerosol. This work shows a preliminary in vitro biochemical characterisation of an A1PI concentrate before and after aerosolisation.
Human A1PI was aerosolised using a commercial nebuliser system. The aerosolisation of 10 ml of A1PI solution took approximately 20 minutes. Three lots were analysed before and after nebulisation for: temperature, elastase inhibitory activity (chromogenic), protein (Bradford), pH, osmolality, turbidity, molecular distribution (HPLC) and SDS-PAGE.
Aerosol formation does not increase the solution temperature and results of pre- and post-aerosolised product do not show variations as refers to elastase inhibitory activity (21.3±0.8 mg active A1PI/ml and 20.8±0.8 mg active A1PI/ml, respectively), protein (16.8±1.1 mg/ml and 16.4±1.4 mg/ml, respectively) and monomers (81.5±3.2% and 82.7±2.5%, respectively). No fragments are noticed. Results for pH, turbidity and osmolality remain unchanged as well. The determination of molecular weight by SDS-PAGE
test shows a band corresponding to the A1PI molecular weight in both instances.
Aerosolised A1PI is quickly formed and no increase of temperature or alterations in the parameters studied are observed. The biochemical activity is preserved.

- **Characterisation and stability of a new alpha-1 proteinase inhibitor concentrate.**

*M. López, M. José, M. Costa, M. Massot, J.I. Jorquera*
*Research and Development Area, Instituto Grifols S.A. Barcelona, Spain.*

An extensive biochemical characterisation and stability study of a new alpha-1 proteinase inhibitor (A1PI) concentrate was performed.
Six lots were studied for: pH, turbidity, protein (Bradford), water content, solubility, elastase inhibitory activity (chromogenic), specific activity, molecular distribution (HPLC), electrophoresis (CAME), accompanying proteins (immunonephelometry), SDS-PAGE, Immunoblot, isoelectric focusing (IEF). In order to assign shelf-life, 6 lots (0.5 g and 1 g) were studied at different temperatures (5±3 ºC, 30±2 ºC and 40±2ºC).
The product shows an elastase inhibitory activity of 548±48 mg active A1PI/vial of 0.5 g and the specific activity is 1.2 mg active A1PI/mg protein. The A1PI monomer percent is 82±2% and purity is 89±2%. SDS-PAGE and Immunoblot studies confirm the high purity of the product. The IEF analysis shows isoelectric points between 4 and 5 (A1PI isoforms). Finally, small amounts of albumin, IgA and transferrin can be detected. The product does not show instability signs after 3 years of storage between 2 ºC and 30 ºC.
The product studied is a lyophilisate containing 20 mg of active A1PI/ml after reconstitution, has a high purity level and functionality against elastase, and shows a remarkable stability profile.

- **Circulating monocytes in healthy and emphysema individuals with and without a1-antitrypsin deficiency.**

*R. Dominaitiene, S. Janciauskiene*
*Department of Medicine, Wallenberg laboratory, University Hospital Malmö, Malmö, Sweden.*

Chronic obstructive pulmonary disease (COPD) is characterized by incompletely reversible airflow obstruction associated with inflammation in the lungs. Inflammatory cells, such as neutrophils, monocytes, eosinophils, and lymphocytes, are all known to participate in inflammatory processes associated with COPD. The only known genetic factor related to COPD, is inherited deficiency of a1-antitrypsin (AAT), an inhibitor of serine proteases. We investigated the properties of blood monocytes isolated from sex and gender matched healthy (n=30) and emphysema (n=20) individuals with and without AAT deficiency. Monocytes were cultured alone or with LPS (1mg/ml) for 18 h and the release of pro-inflammatory molecules was analysed by ELISA methods. Monocytes isolated from emphysema patients release significantly more gelatinase B (MMP-9) (2.5-fold), but less TNFa (2.3-fold) and IL-8 (1.8-fold) compared to monocytes obtained from healthy individuals. In addition, LPS-activated cells from emphysema and healthy subjects release higher quantities of IL-6 and MCP-1 or MMP-9 and ICAM-1, respectively, compared to non-activated cells. Independently on disease status, monocytes from ZZ-AAT carriers release higher amounts of TNFa (by 4.4-fold) than MM-AAT. LPS-activated monocytes from ZZ-AAT carriers release more IL-8 (3.3-fold), while MM-AAT-monocytes release more ICAM-1 (3.4-fold), compared to non-stimulated cells. These data provide evidence that properties of circulating monocytes in healthy and emphysema subjects with normal M and deficiency Z-AAT variant are not homogeneous, and respond differently when exposed to pro-inflammatory stimulus. These findings may add new insights into better understanding inflammation in COPD.

- **Cytokines in the pathogenesis of Chronic Obstructive Pulmonary Disease. What is really important?**

*J.M. Saffeniave[1], P.T. Reid[2]*
*[1]Rayne Laboratory, Centre for Inflammation Research, Edinburgh University Medical School,*
*[2]Western General Hospital, Edinburgh, Scotland, UK.*

There is growing recognition that chronic obstructive pulmonary diseases (COPD) have an inflammatory component that is central to the development and progression of the illness and contributes to many of the extra-pulmonary features. Cytokines are secreted locally by inflammatory and structural cells and orchestrate the inflammatory and immune response. Although their role has generated significant interest in the pathogenesis of asthma and other inflammatory lung diseases, it is only recently that cytokines have attracted attention in COPD, implicating a variety of inflammatory cells as effectors in the pathogenesis of the disease (innate immunity cells and lymphocytes). Clinically, the course of COPD may be complicated by inflammatory exacerbations, resulting in increased levels of pro-inflammatory cytokines (TNF, interleukin-8...). In addition, some investigators have suggested that a systemic component may be present in COPD, with elevating levels of circulating soluble TNF receptor sTNF-R55 and IL-6 and IL-8 levels, as well as increased levels of the acute phase reactants C-reactive protein and lipopolyscaccharide binding protein (LBP). In addition to these clinical data, recent animal model studies have implicated inflammatory cytokines such as TNF, as well as type 1 (IFN-g), type 2 (IL-13) or growth factors (PDGF, TGF-a,VEGF) in the modulation of the emphysematous phenotype.

- **Engineered Liver Stem Cell Transplantation for Alpha-1 Antitrypsin Deficiency.**

*Sihong Song, Rafal Witek, Yuanqing Lu, Bryon E. Petersen, Terence R. Flotte*
*Departments of Pharmaceutics, Pathology and Pediatrics, University of Florida, Gainesville, Florida, USA.*

In a subset of patients homozygous for the PI*Z mutation, liver disease may develop, apparently related to polymerization of the mutant protein within the endoplasmic reticulum of affected hepatocytes. Gene therapy for AAT deficiency-related liver disease will likely be dependent upon transduction of a high percentage of hepatocytes. Ex vivo gene delivery to liver stem cells followed by transplantation has great potential for increasing the rate of long-term transduction. In order to optimize the transduction efficiency of rAAV vectors, rat liver stem cells were infected with five serotypes of rAAV- CB-hAAT vector (type 1, 2, 3, 4 and 5). Results from this experiment showed that rAAV1 mediated the highest hAAT secretion into the culture medium. The transgene expression can be clearly detected at 4 days after infection with AAV1 (supernatant AAT concentration of 15 mcg/ml), while no detection obtained until 10 days after infection with AAV2, 3 and 5. AAV4 did not mediate any! transgene expression in this study. Subsequently, liver stem cells from C57 bl/6 mice were transduced with the rAAV1-CB-AAT vector at 50,000 MOI for 2h.
After transducion cells were washed and resuspended in saline solution at appropriate concentration to give approximately 1x106 cells per 100$\mu$l. Transduced liver stem cells were transplanted into the liver of partial hepatectomized C57bl/6 recipients (1x106 cells/mouse,

n=6). The transgene expression was monitored by measuring the serum level of hAAT. Two weeks after transplantation, hAAT were detected from recipient mice. The expression levels (1 to 4 g/ml) were sustained at least 6 weeks after stem cell transplantation. These results indicate that AAV1 infect liver stem cells more efficient than other serotypes, and that ex vivo manipulation and transplantation of liver stem cells provide an alternative approach for liver gene therapy.

*Supported in part by grants from NIH (DK58327, HL59412), the Alpha One Foundation, and the Juvenile Diabetes Research Foundation.*

- **Factors associated with the development of pulmonary emphysema in smokers.**

*F. Casas[1], M. Contreras[1], O. Carazo[2], A. Cabrera[3], B. Lara B[4]*
*Dpt of Pneumology and [2]Radiology, HU San Cecilio, Granada, [3]Escuela Andaluza de Salud Pública, [4]Dpt. Pneumology. Hospital Clínic i Provincial. Institut de Pneumologia i Cirurgia Toràcica, Barcelona. Spain.*

OBJECTIVES: To analyse the factors associated with the development of pulmonary emphysema (PE) in current and former smokers of ³20 cig/day over at least 15 years.
METHODS: A transversal study was performed in 78 subjects (74 males) of 52.9±1.2 years, with a BMI of 24.9±0.4, being active smokers and 24 ex-smokers of 31±1.1 cig/day over 27.2±1.0 years. PE by PFP was defined by KCO£85% with RV³135% and/or Csp³0.08. PE by TACAR was defined by a visual evaluation scale as a percentage of low attenuation areas (LAA).
RESULTS: Of the 78 subjects included 79.5% presented dyspnoea, which was clinically relevant in 32.1%. Serum concentrations of a1 antitrypsin (a1AT) was 145.0±3.9 mg/dl and a1AT was>120 in 82.1% and between 85-119mg/dl in 17.9% with no significant differences observed between the two groups for COPD or PE. Multivariate logistic regression showed that younger subjects, longer smoking time, advanced clinical stage and lower PaO2 had a greater probability of PE (PFP and /or TCAR).
CONCLUSIONS:
1. younger smokers, longer smoking time, advanced clinical stage and lower PaO2 have a greater probability of developing PE (PFP and/or TACAR).
2. Mild a1AT deficiency was not a determining factor for COPD or PE.





- **Genotypical identification of alpha 1-antitrypsin variants by SexAI/Hpy99I RFLP.**

*Michele Zorzetto[1], Ilaria Ferrarotti[1], Roberta Scabini[2], Paola Mazzola[1], Eliana Disabella[2], Ilaria Campo[2], Maurizio Luisetti[1]*
*[1]Laboratorio di Biochimica e Genetica. Clinica di Malattie dell'Apparato Respiratorio. IRCCS Policlinico S. Matteo, Pavia and [2]Laboratorio di Immunogenetica, Dipartimento di Genetica e Microbiologia, Università di Pavia, Italy.*

There is worldwide growing awareness an alpha1-antitrypsin deficiency (AATD), a major hereditary disorder in Caucasian. The gold standard for laboratory diagnosis of AATD is thin-layer isoelectrofocusing, which is however labour intensive and should be performed in reference laboratories. The aim of this study was to find an easy, fast, and cheap method for detecting a1 antitrypsin S and Z variants, the most frequent variants associated with AATD. The novel method herein described is based on SexAI/Hpy99I RFLP. We studied samples from 20 subjects enrolled in the Italian National Registry for AATD, previously typed by isoelectrofocusing. In order to check the results obtained by the SexAI/Hpy99I method, all samples were also submitted to genotyping by other reported methodologies (TaqI RFLP, direct sequencing and an amplification-reverse hybridization commercial kit) that are more expensive or requiring longer performance time. We found a complete agreement among genotyping obtained with these methods. We concluded that this novel method combines efficiency, ease, swiftness, and low costs.

- **GSTP1 polymorphisms in patients with COPD and alpha-1 antitrypsin deficiency.**

*C. De la Roza, R. Vidal[1], F. Rodríguez-Frías[2], X. Costa[2], S. Vilà, B. Lara, R. Jardí[2], M. Miravitlles Pneumology Dept., Hospital Clínic, Barcelona, [1]Pneumology Dept and [2]Biochemistry Dept, Hospital Vall d'Hebron, Barcelona, Spain.*

Glutathion S-transferase P1 (GSTP1) is related with the detoxification of potentially carcinogenetic aromatic policyclic hidrocarbures present in tobacco smoke. Recent studies suggest that polymorphism conditioning the expression of the slow enzyme form (Val 105) is related with an increased risk of developing COPD.
The objective was to study the frequency of the slow mutation Val105 in a population of moderate to severe COPD patients and in patients with alpha-1 antitrypsin deficiency (Pi ZZ).
We studied a group of 99 patients with a moderate-severe COPD (FEV1< 50% and < 60 years old) and 50 patients with alpha-1 antitrypsin deficiency (Pi ZZ). The control group consisted of 198 unrelated healthy subjects. GSTP1 genotyping was performed by a novel real-time polymerase chain reaction (PCR) amplification assay using the LightCycler high-speed thermal cycler.

Distribution of GSTP1 polymorphisms in the three studied populations.

| | Ile/Ile wild | Ile/Val | Val/Val | P* vs control | P* vs PiZZ |
|---|---|---|---|---|---|
| COPD (n=99) | 53 (50) | 36 (34) | 10 (9.4) | 0.30 | 0.022 |
| PiZZ (n=50) | 15 (29.4) | 26 (51) | 9 (17.6) | 0.008 | |
| Control (n=198) | 97 (46) | 88 (41.7) | 13 (6.2) | | |

Clinical and functional parameters classified according to the GSTP1 genotypes.

| | COPD (n=99) | | | PiZZ (n=50) | | |
|---|---|---|---|---|---|---|
| | Ile /Ile wild (n=53) | Ile/Val (n=36) | Val/Val (n=10) | Ile/Ile wild (n=15) | Ile/Val (n=26) | Val/Val (n=9) |
| Age (years) | 57.36 (9.29) | 55.81 (9.01) | 55 (5.75) | 43.82 (13.15) | 47.43 (12.5) | 45.4 (14.31) |
| Pack-years | 49.83 (18.7) | 50 (26.13) | 59.8 (26.8) | 18 (17.64) | 27.5 (17.03) | 17.57 (8.44) |
| FEV1 (%) | 37.9 (13.8) | 35 (11) | 40.5 (7.4) | 81.1* (32.7)* | 45.4 (32.9) | 55.9 (46.7) |

ANOVA* p= 0003

The frequency of the slow variant of GSTP1 (Val 105) is significantly increased in patients with alpha-1 antitrypsin deficiency (Pi ZZ) but not in patients with COPD.
This mutation appears to be related with worse lung function in the PiZZ population. In contrast, no influence on FEV1 was observed in patients with COPD and normal AAT serum concentrations.

*Supported in part by a grant from Fundació La Marató de TV3 and a grant Maria Ravà of FUCAP.*

- **Identification of promoters controlling alpha-1 antitrypsin gene expression in islet cells.**

*Sihong Song and Ge Zhou*
*Department of Pharmaceutics, College of Pharmacy University of Florida. Gainesville, Florida, USA.*

Alpha-1 antitrypsin is a serine proteinase inhibitor and has anti-inflammatory property. The majority of the serum AAT is secreted from liver, where the gene is controlled by a liver-specific promoter. In macrophage, AAT gene is driven by a macrophage-specific promoter localized 2 kb upstream of the liver-specific promoter and transcribed into two specific AAT mRNAs. In this study, we have examined the AAT gene expression in human islets. Fresh human islets were isolated and cultured for 10 days.
Accumulation of AAT in culture medium was detected by ELISA. AAT mRNA was detected by RT-PCR. To identify promoters, that control the gene expression in human islet, we have performed 5'-RACE analysis. Results from this study showed that AAT gene expression in islet is controlled by at least four promoters. Although the liver-specific promoter appears to be the major promoter, macrophage-specific promoter is also active in human islets. In addition, we have identify two new promoter! s and new alternative splicing patterns within exon 1A, 1B, 1C and exon 2 region. These data suggest that AAT gene is regulated by multiple systems in islet cells and may imply special functions of AAT in protecting islets.

*Supported in part by grants from NIH (DK62652), the Alpha One Foundation, and the Juvenile Diabetes Research Foundation.*

- **Intravenous alpha-1 antitrypsin administration induces cyclic changes in pulmonary compliance in patients with emphysema due to alpha-1 antitrypsin deficiency.**

*F. Casas, M.R. Cabello, M. Gallardo, A. Martinez[1], M. Miravitlles[2], B. Lara[1], A. Mengibar, R. Soto*
*Pneumology Dpt. University Hospital San Cecilio. Granada, Spain. [1]Pneumology Dpt. Hospital Torrecardenas. Almería, Spain. [2]Pneumology Dpt. Hospital Clinic i Provincial. Institut de Pneumologia i Cirurgia Toràcica. Barcelona, Spain.*

INTRODUCTION: It has been observed that different patients with pulmonary emphysema (PE) due to alpha-1 antitrypsin deficiency (AATD) undergoing an endovenous substitution regimen every 21 days present improvement in dyspnoea which begins on days 4-5 postinfusion, intensifying on days 8-10 and begins to remit after day 14.
OBJECTIVE: The objective was to ascertain whether improvement in dyspnoea in these patients is related to changes in lung function tests or pulmonary mechanics attributable to AAT infusion.
METHOD: Four patients with emphysema due to AATD were prospectively studied during 10 cycles of AAT infusion at doses of 180mg/kg every 3 weeks. Serum AAT concentrations were determined and pulmonary function test (FVC,FEV1 FRC, RV/TLC, DLCO/VA and Cst-E-expiratory static pulmonary compliance) performed before (phase 0), day 5 (phase 1) and day 10 after AAT infusion (phase 2).
RESULTS: All patients presented cyclic changes in Cst-E during the 10 infusion cycles studied consisting in a significant increase in Cst-E values which, on some occasions, peaked on day 5 ($p<0.01$) and, on others, on day 10 ($p<0.001$), and in FRC ($p<0.05$ in phase 1; $p=0.08$ in phase 2) with no significant modifications in the RV/TLC ratio or in FEV1. Our results suggest that AAT infusion at a dose of 180mg/kg every 21 days induces cyclic alterations in pulmonary function manifested by an increase in Cst-E and FRC achieving maximum values between days 5 and 10 of infusion.
CONCLUSIONS:
1.AAT infusion at a dose of 180mg/kg every 21 days induces cyclic alterations in pulmonary function tests.
2. These changes are reported as clinical improvement in dyspnoea of unknown cause, which may be related to quantitative and/or qualitative modifications in the pulmonary surfactant.

- **Regulation of alpha-1-antitrypsin (AAT) gene expresson in human alveolar epithelial cells (A549).**

*Kevin Morgan, Sally Plummer, Stephen Morley, Noor Kalsheker*
*Institute of Genetics. University of Nottingham. Nottingham, England, UK.*

The liver produces the bulk of circulating AAT but other tissues contribute significantly to local concentrations; expression in the lung can be increased by cytokines up to 100-fold and AAT may play a beneficial role in combating the destructive effects of neutrophil elastase. A549 cells utilise the AAT monocyte transcription initiation site so we have mapped functional activity in this region using luciferase reporter gene assays. The minimal promoter was mapped to a construct that contained the proposed TATA box and 8 SP1 sites. Site-directed

mutagenesis of specific sites within this region demonstrated that the proposed TATA box was indeed functional in A549 cells as were individual SP1 sites. The introduced point mutations all significantly reduced reporter gene activity by at least 60%. We have also obtained evidence that there is cytokine induced enhancer activity (4 to 6-fold over basal) in the region extending 9kb upstream of the monocyte promoter which together with changes in the stability of AAT mRNA following cytokine stimulation could explain the large increases seen in protein levels. These studies highlight significant differences in which the AAT gene is regulated in the liver and lung.

*This work was supported in part by the Eurolung Consortium - QLG1-CT-2001-01012.*

- **Viral safety of an alpha 1 proteinase (A1PI) inhibitor therapeutic concentrate.**

*Hermínia Biescas, Pilar Ruiz, Rodrigo Gajardo, Pere Ristol, Marta Massot, Juan I. Jorquera*
*Research & Development Area, Instituto Grifols, S.A. Barcelona, Spain.*

Pathogen transmission has been a concern in the past for some plasma derived therapeutic concentrates. We present the strategy followed to maximize the safety of an A1PI concentrate, and the experimental results from viral elimination procedures applied to the concentrate.
Plasma is obtained from healthy donors complying with current regulations concerning blood and plasma donation, including serological testing for HIV and Hepatitis B and C viruses (HBV, HCV). Further, plasma is tested by Nucleic Acid amplification technologies (NAT) for those viruses and for Parvovirus B19. Hepatitis A virus (HAV) NAT is currently being introduced. Moreover, specific elimination steps (Solvent-Detergent treatment -SD- and Nanofiltration through 15 nm pore size filters) are included in the production process to eliminate potential residual risks.
The efficacy of the elimination methods is measured through experimental inoculation of viruses into laboratory scale processes. These experiments showed 5.4 log10 reduction of the viral load during SD for an enveloped model virus and about 4 log10 reduction during the Nanofiltration step for HAV and porcine parvovirus. Therefore, the overall reduction capacity for enveloped viruses can be estimated to be higher than 9 log10.





## Meeting site

WORLD TRADE CENTER
GRAND MARINA HOTEL
Moll de Barcelona, s/n
08039 Barcelona (Spain)

## Language

English will be the official language.

## PowerPoint projection

Facilities will be available for single PowerPoint projection. For any other kind of technical support, please contact the Organizing Secretariat prior to the meeting.

## Open Session

Oral presentations are scheduled on June 13th from 3 pm to 4 pm. The allotted time will be 15 minutes (10' presentation + 5' discussion).

## Poster presentation

The exhibit of posters will start on June 12th at 9 am.
Poster (cm90 wide x cm120 high) will be exhibited in the designated area. The setting up and the removal of poster should be done by the authors. Poster assistants will be present for any information or help. At least one of the authors is required to attend his/her position during the scheduled time (June 12th from 10 am to 10,30 am and June 13th from 10,20 am to 10,45 am).

## Registration fee

| | |
|---|---|
| Delegate | 250 € (VAT included) |
| Accompanying person | 150 € (VAT included) |
| Patient's groups (June 11) | 85 € (VAT included) |
| Social dinner | 80 € (VAT included) |
| Friday dinner | 65 € (VAT included) |

The fee includes: attendance at the scientific sessions, congress-member documentation, attendance certificate, working lunches and coffees. Social dinner and Friday dinner are not included in fee.
The registration and the hotel booking are available in the congress' web site www.bcmedic.com/airmeeting.

# AIR

## Social events

JUNE 10: Welcome Reception Patient's Groups. Grand Marina Hotel (Eighth Floor), 8.30 pm.

JUNE 11: Welcome Reception. Grand Marina Hotel (Eighth Floor), 8.30 pm.

JUNE 12: Social dinner. Casa Batlló (modernist house designed by Gaudí), 8 pm.

JUNE 13: Friday dinner. Rúccula Restaurant, next to the Grand Marina Hotel, 8 pm.

## Accompanying person tour

12 JUNE: In the morning, visit the Barcelona Gothic. Lunch in the Grand Marina Hotel and rest of the day is free. Meeting point at the Grand Marina Hotel's hall at 9 am.

13 JUNE: In the morning, vist the Barcelona Modernist. Lunch in the Grand Marina Hotel and rest of the day is free. Meeting point at the Grand Marina Hotel's hall at 9 am.

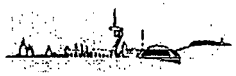
## Climate and clothing

The weather in June is usually sunny. At nights, it is recommended to bring a jacket.

## Variation

The Scientific Committee and the Organizing Secretariat reserve the right to make any change to the meeting program for technical and/or scientific reasons.

## Organising secretariat



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AIR

## The star city of the Mediterranean arc

Mediterranean city. Two thousand years of history. Open to every innovation. Welcoming, plural, diverse. A city to live and share.

This Barcelona that's waiting for you is a city with a Roman subsoil and living in the 21st century, where people speak two languages, Catalan and Castilian, where Gothic architecture stands alongside the most contemporary buildings, where economic dynamism blends with intellectual effervescence, and where the sea is a stage and the mountain its backdrop. Today's Barcelona is living one of its finest moments.

The city has the biggest/widest selection of modernist architecture: a genuine open-air museum. Most of the buildings, built in this unique style, are in the Eixample, a district planned in 1860 by the engineer Ildefons Cerdà which constitutes a unique model of European urban/town planning. The Sagrada Familia, the Casa Batlló, the Casa Amatller and the Casa Milà are some examples of this and Antoni Gaudí one of its most representative architects.

Barcelona is the only European capital with over four kilometres of beaches where you can enjoy the most modern amenities, the beaches are not far form Barcelona's historical and cultural landmarks, and they have opened up our modern and cosmopolitan city to the sea.

Today, the Olympic Harbour and the old port, the Port Vell, are some of the main meeting places, with many bars, restaurants, shops and recreational areas.

Barcelona is, without a doubt, a city of marvels: on foot, by bicycle or bus, it is a magnificent spectacle which you should not miss.

Catalan cuisine, which is typically Mediterranean, is based on natural seasonal produce. Olive oil, vegetables, fruit, fresh fish and seafood, cod, pork in all its variety, many different cakes, and a wide selection of high quality wines and caves, these are the basic ingredients of our diet. The quality of these products and the way they are combined renders our cuisine both rich and healthy, simple and varied, natural and refined. It is straightforward but there is a huge variety; you can find both modern Catalan food and traditional Catalan menus; our cuisine is one of both smooth and contrasting flavours, appetising and inviting. What's more in Barcelona you can also find international cuisine: French, Italian, Japanese, Chinese, fast food, and so on.

Come to Barcelona and take it away in your memory.

Barcelona

# CHARACTERISATION AND STABILITY OF A NEW ALPHA-1 PROTEINASE INHIBITOR CONCENTRATE.

López M, José M, Costa M, Massot M. and Jorquera J.I. Research and Development Area. Instituto Grifols S.A. Barcelona; Spain.

## OBJECTIVE

The human alpha-1 proteinase inhibitor (a1PI) or alpha-1 antitrypsin (AAT) is a serum glycoprotein of approximately 53 kDa. The main substrate is leukocyte elastase derived from neutrophils. Elastase is an enzyme involved in the proteolysis of the connective tissue. A1PI inhibits the activity of elastase produced by the neutrophils to protect the lung. This work presents a wide characterisation of the product, as well as its stability.

## MATERIALS AND METHODS

Six lots were studied for the following parameters: pH, protein (Bradford), water content (Karl Fischer), elastase inhibitory activity (chromogenic), specific activity, molecular distribution (HPLC), electrophoresis (CAME), accompanying proteins (immunonephelometry), SDS-PAGE and isoelectric focusing (IEF).

In order to establish the stability of the product, different parameters were studied on 6 lots at temperatures of 5±3 ºC, 30±2 ºC (3 years) and 40±2 ºC (12 months).

## RESULTS

*Table 1: Characterisation (n=6)*

| Parameter | Average | SD |
|---|---|---|
| Elastase inhibitory activity (mg A1PI/0.5 g vial) | 548 | 48 |
| Protein (g/0.5 g vial) | 0.47 | 0.06 |
| Specific activity (mg A1PI/mg protein) | 1.17 | 0.16 |
| pH | 7.48 | 0.07 |
| Turbidity (NTU) | 4.4 | 0.8 |
| Water content (%) | 0.35 | 0.12 |
| Purity (%) | 89.1 | 6.1 |
| Main peak % (HPLC) | 82.0 | 2.2 |

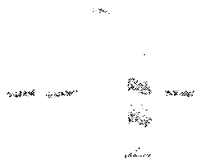


*Figure 1: SDS-PAGE, from left to right: lines 1,2 and 5 A1PI. Lines 3 and 4: high and low molecular weight standards. One main band corresponding to A1PI, of approximate molecular weight 56 kDa, is observed*

*Table 2: Accompanying proteins (n=6)*

| Protein, mg/ml | Average | SD |
|---|---|---|
| IgG | 0.01 | 0.003 |
| IgA | 1.25 | 0.35 |
| Albumin | 0.27 | 0.66 |
| Haptoglobin | 0.12 | 0.14 |
| Transferrin | 0.05 | 0.03 |
| Ceruloplasmin | < 0.018 | -- |

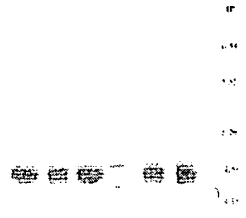


*Figure 2: IEF, from left to right: lines 1,2,3,5 and 6: A1PI. Line 4: IEF standard.*

*Several bands are obtained of A1PI between 4 and 5, similar to the physiological A1PI.*

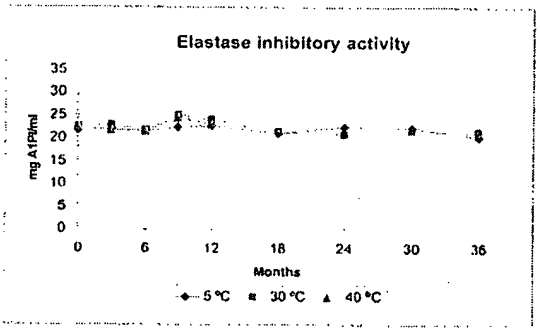



*Figure 3: Evolution of the elastase inhibitory activity.*

### *Stability*

The evolution of the elastase inhibitory activity is plotted in the figure 3. No significant differences are noticed as refers to activity over the period studied.

Other parameters studied such as turbidity, protein, specific activity, water content, etc., do not show variations either, what indicates good stability of the product..

## CONCLUSIONS

***The human A1PI concentrate studied is a lyophilised product with an elastase inhibitory activity of 20 mg/ml after reconstitution.***

***It shows high purity, and an IEF profile comparable to the physiological A1PI.***

***It remains stable for 3 years at temperatures between 2 ºC and 30 ºC.***

**Acknowledgements:** The authors wish to thank Rosa Altes and Carmen Ranera for their technical assistance.

# ALPHA-1 PROTEINASE INHIBITOR: ADJUSTMENT OF INDIVIDUAL THERAPEUTICAL DOSES.

López M, Muñoz N, Costa M, Massot M. and Jorquera J.I. Research and Development Area. Instituto Grifols S.A. Barcelona, Spain.

## OBJECTIVE

- To study the possibility of unifying the alpha-1 proteinase inhibitor (A1PI) content of a number of vials into a single container in order to be able to use large volumes as required for each patient's treatment.
  To study the stability in polypropylene bags, after reconstitution.

## MATERIALS AND METHODS

- Lyophilised product, 0.5 g and 1 g. Solvent: water for injection, 25 ml and 50 ml, respectively.
  Composition per vial (1 g): Active A1PI: 800-1300 mg, Total protein: 1.45 g, Chloride: 107-284 mg, Phosphate: 71-143 mg.
- The filling system is based on the dosage of bags through a sterilizing filter of 0.22 µm. Polypropylene, empty bags with 0.22 µm filter in the filling port, sterilized, available in several volumes.
- The sterile dispenser used is a closed system for sterile volumetric dosage of solutions controlling, unit by unit, the integrity of the filter.
- Analytical methods: Elastase inhibitory activity (chromogenic), protein (Bradford), monomer (HPLC), turbidity (nephelometry), pH and osmolality.
- Design of stability studies: Polypropylene bags, 100 ml capacity, containing 40 ml of reconstituted product (sterile filling system).
  Storage: Temperatures studied: 5 °C, 30 °C/ 60%RH and 40 °C/60%RH. Protected from light. Analytical follow-up at different intervals.

## RESULTS

*Table 1: Stability at 5 °C (mean ± SD)*

| Parameter | 0 days | 7 days | 15 days | 1 month |
|---|---|---|---|---|
| Elastase inhibitory activity (mg/ml) | 20.5 ± 1.2 | 20.2 ± 0.7 | 21.6 ± 2.1 | 22.6 ± 2.0 |
| Protein (mg/ml) | 17.3 ± 2 | 17.6 ± 1.0 | 17.6 ± 0.7 | 18 ± 1.2 |
| Monomer (%) | 82.7 ± 3.0 | 82.5 ± 3.0 | 82.1 ± 2.8 | 82.6 ± 2.6 |
| Turbidity (NTU) | 3.4 ± 0.4 | 3.4 ± 0.3 | 3.3 ± 0.3 | 3.1 ± 0.2 |
| pH | 7.41 ± 0.18 | 7.52 ± 0.01 | 7.51 ± 0.01 | 7.50 ± 0.00 |
| Osmolality (mOsm/kg) | 282 ± 47 | 282 ± 50 | 282 ± 50 | 287 ± 53 |

*Table 2: Stability at 30 °C ( mean ± SD)*

| Parameter | 0 days | 7 days | 15 days | 1 month |
|---|---|---|---|---|
| Elastase inhibitory activity (mg/ml) | 20.5 ± 1.2 | 20.3 ± 2.1 | 20.6 ± 2.6 | 20.9 ± 0.2 |
| Protein (mg/ml) | 17.3 ± 2 | 17.8 ± 1.5 | 17.6 ± 0.8 | 18.1 ± 1.1 |
| Monomer (%) | 82.7 ± 3.0 | 82.0 ± 3.0 | 81.7 ± 2.9 | 81.5 ± 2.9 |
| Turbidity (NTU) | 3.4 ± 0.4 | 3.3 ± 0.2 | 3.3 ± 0.9 | 3.7 ± 0.2 |
| pH | 7.41 ± 0.18 | 7.51 ± 0.00 | 7.51 ± 0.00 | 7.50 ± 0.00 |
| Osmolality (mOsm/kg) | 282 ± 47 | 283 ± 50 | 284 ± 50 | 289 ± 55 |

*Table 3: Stability at 40 °C (mean ± SD)*

| Parameter | 0 days | 7 days | 15 days | 1 month |
|---|---|---|---|---|
| Elastase inhibitory activity (mg/ml) | 20.5 ± 1.2 | 19.7 ± 0.7 | 19.0 ± 0.6 | 17.5 ± 0.9 |
| Protein (mg/ml) | 17.3 ± 2.0 | 17.4 ± 1.7 | 17.3 ± 0.9 | 17.2 ± 1.3 |
| Monomer (%) | 82.7 ± 3.0 | 81.1 ± 2.8 | 78.1 ± 2.9 | 73 ± 2.1 |
| Turbidity (NTU) | 3.4 ± 0.4 | 3.6 ± 0.2 | 4.4 ± 0.6 | 6.9 ± 0.8 |
| pH | 7.41 ± 0.18 | 7.51 ± 0.00 | 7.51 ± 0.00 | 7.50 ± 0.00 |
| Osmolality (mOsm/kg) | 282 ± 47 | 283 ± 50 | 288 ± 53 | 292 ± 53 |

Additionally, after one month at 40 °C, no migration of the plastic additives (Irganox 1010, Irganox 1076, Ethanox 330, BHT) studied to the A1PI solution is observed. A slight decrease in the weight of the semipermeable final containers after 30 days at temperatures higher than 5°C is noticed.

*Figure 1: Dosage of a source A1PI solution from several small-volume vials into a final bag*

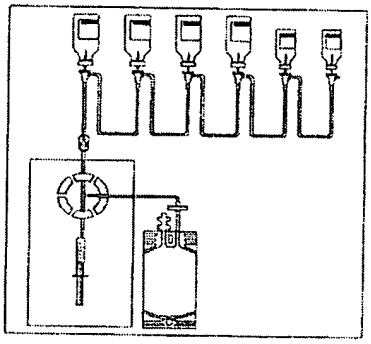
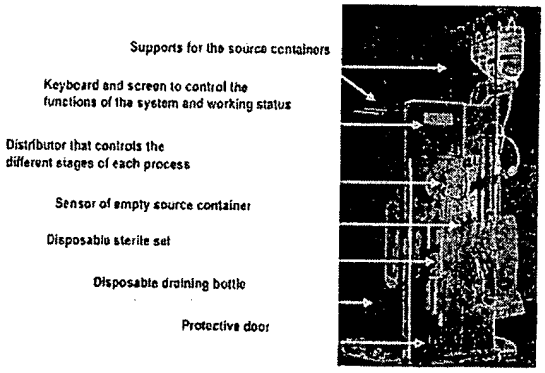


*Figure 2: Filling system*




Acknowledgements: Rosa Allés, Carmen Ranera

## CONCLUSIONS

*Possibility of filling the reconstituted product in polypropylene bags by means of a sterile system, adapting the therapeutical dose to the patient.*

*Stability at 15 days, at temperatures of 5 °C and 30 °C (for the latter, under controlled moisture conditions), obtaining good results. The study at 30 days was performed to provide a safety margin.*

AIR 2003, International Meeting on Alpha-1 Antitrypsin Deficiency. Barcelona, Spain. June 11-13, 2003

# VIRAL SAFETY OF AN ALPHA 1 PROTEINASE INHIBITOR (A1PI) THERAPEUTIC CONCENTRATE.

Biescas H., Ruiz P., Gajardo R., Ristol P., Massot M., Jorquera J. I., Research and Development Area, Instituto Grifols, S.A., Barcelona, Spain.

## INTRODUCTION

Pathogen transmission has been a concern in the past for some plasma-derived therapeutic concentrates. We present the strategy followed to maximize the safety of an A1PI concentrate, and the experimental results from viral elimination procedures applied to the concentrate.

## OBJECTIVE AND METHODOLOGY

Many aspects contribute to the overall safety of the A1PI plasma-derived therapeutic concentrate. Firstly, the source material, plasma, is obtained from healthy donors complying with current regulations concerning blood and plasma donation. Serological testing for HIV, Hepatitis B (HBV) and Hepatitis C (HCV) is required. Furthermore, plasma is tested by Nucleic Acid Amplification Technologies (NAT) for those viruses and for Parvovirus B19. Hepatitis A virus (HAV) NAT is currently being introduced. In addition, current European regulations require fractionation plasma pool testing for HCV RNA using validated NAT procedures. The plasma fractionation pool has to be HCV RNA negative before release. Moreover, the GMP manufacturing production process, which includes specific elimination steps, is likely to eliminate potential virus residual risks. Finally, Pharmacosurveillance contributes, in a last step, to the overall product safety.

A1PI production process includes two specific virus elimination methods: Solvent-Detergent and Nanofiltration through 15 nm pore size. The efficacy of these two methods is evaluated here.

This study reports results of virus elimination experiments performed in accordance with International Guidelines (1,2,3,). The manufacturing steps selected, SD and Nanofiltration, were reproduced at laboratory scale to mimic industrial conditions. Critical parameters were adjusted to evaluate the least favourable conditions for virus removal/inactivation. In particular, the SD concentration was reduced by 50% of regular manufacturing concentration. Three viruses that cover different sizes and physicochemical properties were investigated; Pseudorabies virus (PRV) as a representative for large (120 - 200 nm) enveloped DNA viruses, Hepatitis A (25 - 30 nm) and Porcine Parvovirus (PPV) as a representative of the smallest and most resistant viruses, and also a model virus for the Human Parvovirus B19.

A volume of virus was inoculated into the industrial starting materials and each step was performed in a laboratory model. Residual titers were determined by infectivity assays ($TCID_{50}$; 50% Tissue Culture Infectious Dose). Where no virus was detected a theoretical minimum detectable level (MDL) was used based on a 95% Poisson distribution probability (limit of detection for the assay).

## RESULTS

*Overall Reduction capacity of virus elimination studies for the A1PI manufacturing process*

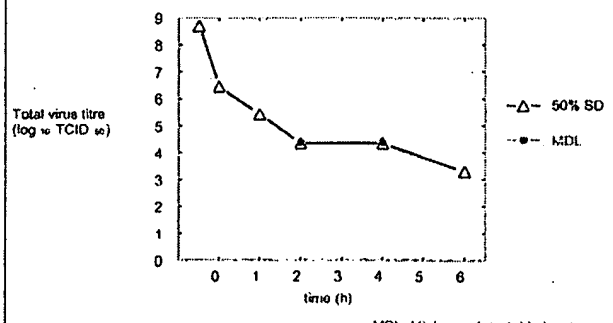
*Reduction Factor (RF) expressed in $log_{10}$ $TCID_{50}$*

| REDUCTION FACTOR (log10) / STEP | ENVELOPED VIRUSES | NON-ENVELOPED VIRUSES | |
|---|---|---|---|
| | PRV (120 - 200 nm) | HAV (27 - 30 nm) | PPV (18-24 nm) |
| SOLVENT DETERGENT (50% of regular concentration) | 5.4 | NA | NA |
| NANOFILTRATION | ≥4.0* | ≥4.0 | ≥4.0 |
| OVERALL REDUCTION FACTOR | ≥9.4 | ≥4.0 | ≥4.0 |

* Underestimated result. Assumed from the smallest viruses (PPV, HAV). Clustering the titers against the smallest viruses gives an estimation of removal for the largest viruses.

*Kinetics of Inactivation*

*at 50% dilution of Solvent Detergent reagent for an enveloped model virus (PRV)*




MDL: Minimum detectable level

## DISCUSSION

**The virus validation experiments of two steps of the manufacturing process of the A1PI concentrate showed 5.4 $log_{10}$ reduction of viral load during the Solvent-Detergent treatment for an enveloped model virus (Pseudorabies) and about ≥4.0 $log_{10}$ reduction during the Nanofiltration step for the smaller viruses HAV and Porcine Parvovirus. Therefore, the overall reduction capacity for enveloped viruses can be estimated to be higher than 9 $log_{10}$ of virus load, thus confirming the extensive experience that the SD mechanism highly contributes to the safety of plasma derivatives. The effectiveness of the nanofiltration through Planova-15 filters removing viruses larger than 15 nm is also evidenced. In addition, other purification steps may also contribute toward the overall safety of the product.**

## REFERENCES

[1] CPMP/268/95. Final. The design, contribution and interpretation of studies validating the inactivation and removal of viruses.

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[3] CPMP/ICH/295/98. "Quality of Biotechnological products: Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin".

*Acknowledgments; The authors thank S. Nieto, N. Domingo, C. Lombart, C. Viera, A. Gil for their expert technical assistance.*

*This project has been partially supported by a grant from the Spanish Ministry of Science and Technology.*

**AIR 2003, International Meeting on Alpha-1-Antitrypsin Deficiency. Barcelona, Spain. June 11-13, 2003.**